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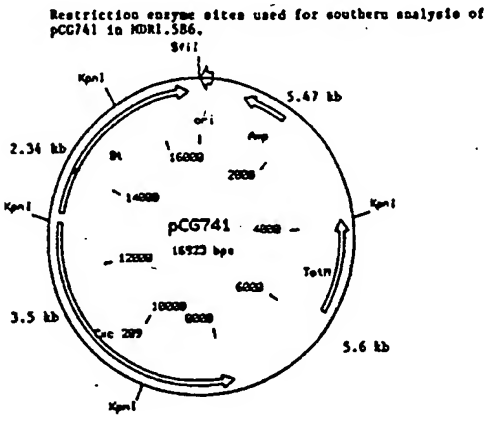
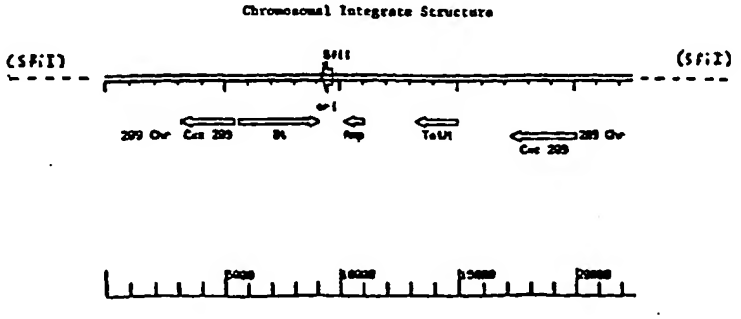
(57) Abstract

Hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with plants are disclosed. The microorganisms are prepared by combining genetic material from an agricultural-chemical-producing microorganism and a plant infecting microorganism to form hybrid microorganisms and selecting from the hybrid microorganisms those hybrid microorganisms which are capable of producing an agricultural chemical, which do not create manifestations of disease in the plant host, and which are capable of entering into an endosymbiotic relationship with the plant host. Methods of use are also disclosed.





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AGRICULTURAL-CHEMICAL-PRODUCING ENDOSYMBIOTIC MICROORGANISMS AND METHOD OF PREPARING AND USING SAME

DESCRIPTION

Agricultural-Chemical-Producing Endosymbiotic Microorganisms and Method of Preparing and Using Same

This application is a continuation-in-part of application Serial No. 07/919,375, filed July 29, 1992, which is a continuation of application Serial No. 07/466,465, filed January 16, 1990, which is a continuation-in-part of application Serial No. 07/266,232, filed October 28, 1988, which is a continuation of application Serial No. 06/799,999, filed November 20, 1985, now abandoned, which is a continuation-in-part of application Serial No. 06/534,071, filed September 20, 1983, now abandoned in favor of continuation application Serial No. 07/166,819, filed March 3, 1988, which itself is a continuation-in-part of application Serial No. 06/484,560, filed April 13, 1983, abandoned in favor of continuation application Serial No. 06/933,337, filed November 20, 1986, and now abandoned in favor of continuation application Serial No. 07/266,221, filed October 28, 1988.

The present invention relates to agricultural-chemical-producing microorganisms, particularly agricultural-chemical-producing bacteria capable of entering into endosymbiotic relationships with host plants whereby the bacteria provide some or all of the plant's agricultural-chemical requirements.

BACKGROUND OF THE INVENTION

Modern commercial agriculture is heavily dependent on the use of agricultural chemicals to enhance the performance of plants. Most notably, chemical fertilizers, particularly fixed-nitrogen fertilizers, are the most common limiting nutrient in crop productivity and often are the most expensive single input for the farmer. Other agricultural chemicals are widely used at great expense to kill plant pests, to prevent disease, or to improve the growing environment of the plant. Still other agricultural chemicals may be added to growing plants or harvested crops to diminish or enhance natural properties or to alter or improve the appearance or sensory appeal of the plant or crop. Agricultural chemicals include, as well, natural or synthetic plant growth regulators, including hormones and the like. Such chemicals are well known to those having ordinary skill in the agricultural art and are hereinafter generically referred to as "agricultural chemicals."

Microorganisms, such as bacteria, fungi, and algae are a natural source of agricultural chemicals. For example, leguminous plants, such as soybeans, alfalfa and clover, derive some of their fixed nitrogen requirements through symbiotic relationships with bacteria of the genus Rhizobium. Particular species of Rhizobium infect the roots of leguminous plants forming nodules in which the bacteria are shielded from oxygen and provided with carbohydrate nutrients. In this anaerobic process, the rhizobia fix atmospheric nitrogen, which is then available for use by the plant.

Microorganisms have also become sources of agricultural chemicals synthesized or prepared by man and applied by spraying or the like onto fields, growing plants, or harvested crops. Antifungal antibiotics, antibacterial antibiotics, and insecticidal antibiotics, for example, have been produced by various bacteria. Antifungal antibiotics include blasticidin S which is produced by Streptomyces griseochromogenes, kasugamycin which is produced by

Streptomyces kasugaensis, and polyoxins which are produced by Streptomyces cacaoi var. asoensis. Antibacterial antibiotics include streptomycin produced by Streptomyces griseus, and tetracycline produced by Streptomyces viridifaciens. Insecticidal antibiotics include tetranactin, produced by Streptomyces aureus strain S-3466, and the bets-exotoxin and delta-endotoxins produced by Bacillus thuringiensis. See K. Aizawa, "Microbial Control of Insect Pests," and T. Misato and K. Yoneyama, "Agricultural Antibiotics" in Advances in Agricultural Microbiology, N.S.S. Rao, Editor (1982), which is specifically incorporated herein by reference in its entirety.

Similarly, microorganisms have become a source of antibiotics for herbicide use. For example, cycloheximide, produced by Streptomyces griseus, and herbicidin A and B, produced by Streptomyces saganoensis, exhibit herbicidal activity. See Y. Sekizawa and T. Takematsu, "How to Discover New Antibiotics for Herbicidal Use" in Pesticide Chemistry, J. Miyamoto and P.C. Kearney, Editors (1983), which is specifically incorporated herein by reference in its entirety.

Moreover, microorganisms are known to produce plant growth regulating substances such as various vitamins, auxins, cytokinins, gibberellin-like substances and other stimulating or inhibiting substances. Brown, in her work on "Seed and Root Bacterization" cited below, attributes the production of such substances to species of Azotobacter, Pseudomonas and Bacillus, including B. megaterium, and B. subtilis.

Bacteria have been found that produce agents active against a number of invertebrates, such as plant pathogenic nematodes. One of these agents is Streptomyces avermitilis, which produces avermectins. S. avermitilis and the antiparasitic compounds it produces are described in U.S. patents 4,310,519, 4,429,042, and 4,469,68, which are specifically incorporated herein by reference in their entirety.

Microorganisms have also been found to produce antiviral antibiotics, including laurusin, which has been isolated from Streptomyces lavendulae, and miharamycin, which has been isolated from Streptomyces miharuensis. See T. Misato, K. Ko, and I. Yamaguchi, "Use of Antibiotics and In Agriculture" in Advances in Applied Microbiology, Vol. 21, (1977), which is specifically incorporated hereby by reference in its entirety.

Other types of bacteria are known to have the ability to solubilize phosphate that is otherwise unavailable to plants. These include those bacteria belonging to the genera Bacillus and Pseudomonas. Attempts to utilize this ability to these bacteria by inoculating seeds or plants with the bacteria have produced inconsistent results. See N.S.S. Rao, "Phosphate Solubilization by Soil Microorganisms" in Advances in Agricultural Microbiology, N.S.S. Rao, Editor (1982), which is specifically incorporated herein by reference in its entirety.

Few symbiotic associations between agricultural-chemical-producing microorganisms and major crop species, such as wheat and corn, have been described in the scientific literature. The development of symbiotic relationships between such microbial species and various crops would have many advantages over man made synthesis and application of the chemical. The advantages of such associations are particularly apparent in the areas of insecticides and fixed-nitrogen fertilizers, which are hereinafter discussed in detail as exemplary of the advantages attainable through the practice of the present invention. It should be understood however, that the teachings contained herein regarding bacterial production of insecticides and of fixed-nitrogen fertilizers may be extrapolated by those having ordinary skill in the art to other bacterially or microbially produced agricultural chemicals, such as those discussed above.

A particular advantage of the present invention is that the agricultural chemical is introduced within the entire plant or parts thereof rather than just on the surface as in

the case when certain agricultural chemicals are sprayed on the plant. For example, insecticides that are sprayed on and do not penetrate into the plant can be ineffective or of only limited effectiveness against insects that bore into the plant. However, the insecticide should be effective when applied as a hybrid, endosymbiotic microorganism of the present invention.

It is estimated that development of a symbiotic relationship between nitrogen-fixing bacteria and major crop species capable of providing even a minor fraction, e.g., 10-20%, of the fixed nitrogen requirements of non-leguminous crop plants, would produce an economically important substitution of photosynthetically-derived energy for fossil fuel energy now expended for production of nitrogen fertilizer. Currently, the major sources of fixed nitrogen for non-leguminous economic crop plants are man-mad products such as anhydrous ammonia, inorganic nitrogen salts and synthetic organic nitrogen compounds. Present annual consumption of nitrogen fertilizer in the United States is estimated to be 12 million tons. Such nitrogen fertilizers are largely prepared by energy intensive processes for the conversion of atmospheric nitrogen into ammonia.

A major thrust of nitrogen-fixation research in recent years has been directed towards the isolation, characterization, transformation and expression in plants of the nitrogen-fixing genes from rhizobia and other nitrogen-fixing bacteria by genetic modification of the plants themselves. This technique requires a complete understanding of the genetics and biochemistry of nitrogen fixation which, it is estimated, is 10 to 15 years or more in the future. See generally, J.E. Beringer, "Microbial Genetics and Biological Nitrogen Fixation," in Advances In Agricultural Microbiology, N.S.S. Rao, Editor (198), and G.P. Roberts et al., "Genetics and Regulation of Nitrogen Fixation," Ann. Rev. Microbiol., 35:207-35 (1981), both of which are specifically incorporated herein by reference in their entirety.

Other types of bacteria besides Rhizobium are known to have the capability of fixing atmospheric nitrogen. These include those belonging to the genera Azotobacter, Azomonas, Derxia and Beijerinckia, which fix nitrogen aerobically, and those belonging to the genus Klebsiella which, like Rhizobium, fix nitrogen anaerobically. The literature is filled with reports of unsuccessful attempts to develop symbiotic relationships, similar to Rhizobium-legume relationships, between these bacteria and non-leguminous plants.

In the early 1940's, for example, it was proposed in the Soviet Union and elsewhere that nitrogen-fixing bacteria could be used as soil inoculants to provide some or all of the fixed nitrogen requirements of non-leguminous crop plants. Indeed, claims of increased crop yield associated with such inoculations were widespread. Critical analysis of the early yield results, however, revealed that positive significant effects occurred in only about one-third of the trials. It was later established that yield increases associated with bacterial inoculation, including inoculation with species of Azotobacter, were primarily due to changes in the rhizosphere microbial population, disease suppression by the inoculants, production of plant growth-promoting substances, and mineralization of soil phosphates. No ability to establish an associative symbiotic relationship with nitrogen-fixing bacteria in non-leguminous plants had been demonstrated. See M.E. Brown, "Seed and Root Bacterization", Annual Review of Phytopathology, 12:181-197 (1974), which is specifically incorporated herein by reference in its entirety.

It has been demonstrated that an artificial symbiosis can be created between non-leguminous plant cells in tissue culture and aerobic nitrogen-fixing bacteria such as Azotobacter vinelandii. The demonstration involved forced association between higher plant and bacterial cells and did not provide a technique whereby a symbiotic association between such nitrogen-fixing bacteria and growing plants

could be created. See P.S. Carlson, et al., "Forced Association Between Higher Plant and Bacterial Cells In Vitro," Nature, Volume 252, No. 5482, pp. 393-395 (1974), which is specifically incorporated herein by reference in its entirety.

Another effort to create higher organisms capable of fixing their own nitrogen, for example, involved attempts to create new organelles in higher organisms by similar forced association of lower forms of organisms having nitrogen-fixing abilities. Such efforts to parallel the theoretical biological development of known organelles have included, for example, the efforts of Burgoon and Bottino to induce the uptake of nitrogen-fixing Gloecapsa sp. blue-green algae by plant cells, the efforts of Davey and Cocking to induce the uptake of nitrogen-fixing Rhizobium sp. bacteria into plant cells, and the efforts of Giles to induce uptake of nitrogen-fixing Azotobacter vinelandii bacteria by ectomycorrhizal fungi. See A.C. Burgoon and P.J. Bottino, Journal of Heredity, Volume 67, pp. 223-226 (1972); M.R. Davey and E.C. Cocking, Nature, Volume 239, pp. 455-456 (1972); and K.L. Giles, "The Transfer of Nitrogen-Fixing Ability to Nonleguminous Plants," Plant Cell and Tissue Culture Principles and Applications, W.R. Sharp et al., Editors (1979). Neither the work of Burgoon and Bottino nor the work of Davey and Cocking was successful in generating a nitrogen-fixing higher organism. If the effects of Giles were successful, which has not been conclusively established, they would at best result in fungi with organelle-like structures capable of fixing nitrogen which are capable only of living on the outsides of roots of forest species rather than living endosymbiotically with major crop plants.

Another attempt to create a symbiotic relationship between nitrogen-fixing bacteria and non-leguminous plants involved the screening and selection of plant hybrids in an effort to identify some plant varieties which were capable of associating with aerobic nitrogen-fixing bacteria, such as Azotobacter vinelandii, in the soil. However, those efforts

resulted in the identification of plant species which were only about .5% as active as soybean plants inoculated with rhizobia which were not deemed to be commercially useful. See S.W. Ela, et al., "Screening and Selection of Maize to Enhance Associative Bacterial Nitrogen Fixation," Plant Physiol., 70:1564-1567 (1982), which is specifically incorporated herein by reference in its entirety.

The numerous unsuccessful attempts to create associative symbiotic relationships between nitrogen-fixing bacteria and non-leguminous plants have resulted in the development of numerous techniques for genetic manipulation and analysis. These attempts have also resulted in rather extensive characterization of the genetic makeup of nitrogen-fixing bacteria such as Azotobacter. For example, it has been discovered that mutant strains of Azotobacter vinelandii lacking the nitrogen-fixation gene could be transformed into bacteria having the capability to fix nitrogen by introduction of genetic material from Rhizobium species. See G.P. Roberts, "Genetics and Regulation of Nitrogen Fixation," Ann. Rev. Microbiol., 35:207-35 (1981). Moreover, new techniques for protoplast fusion, which involve promotion of interspecific genetic recombination by fusing two different cells which have had their cell walls removed, were recognized as providing a potentially attractive technique for the production of new strains for commercial purposes without requiring an understanding of the underlying genetics. It was acknowledged in the scientific literature, however, that it has yet to be determined whether or not protoplast fusion can be used for genetic studies of gram-negative nitrogen-fixing bacteria. See J.E. Beringer, "Microbial Genetics and Biological Nitrogen Fixation," Advances in Agricultural Microbiology, N.S.S. Rao, Editor (1982) at 13.

Indeed, experimentation related to protoplast fusion of nitrogen-fixing bacteria has, prior to the present invention, failed to produce evidence that non-pathogenic endosymbiotic relationships between higher plants and fusion products of

nitrogen-fixing bacteria can be produced. For example, Du Qian-you and Fan Cheng-ying, "A Preliminary Report on the Establishment of Nitrogen Fixation System in the Crown Gall of Tomato Plant," Acts Botanica Sinica, Vol. 23, No. 6 (1981), which is specifically incorporated herein by reference in its entirety, described an attempt to effect protoplast fusion between parent strains of Agrobacterium tumefaciens (Pen^r, Ti) and Azotobacter chroococcum (Pen^s, Nif^+). The hybrids were selected for nitrogen fixation and their resistance to penicillin and were thereafter inoculated on the stem of a tomato plant. The inoculation produced crown galls or tumors found to possess nitrogenase activity under aerobic conditions according to the acetylene reduction test. The fusion products of the experiment produced a pathogenic response in the host plant and were not shown to be capable of entering into an endosymbiotic relationship with the plant. Specifically, it could not be ascertained whether the fixed nitrogen being produced in the vicinity of the tumors was being used by the plant as a source of nitrogen. Finally, it could not be determined whether the nitrogenase activity observed was the result of a pathogenic hybrid or of a penicillin resistant mutant of Azotobacter remaining in a mixed inoculum. Moreover, it has now been discovered that the attempts of Du Qian-you et al. to mimic the nodule formation mechanism of the Rhizobium-legume interaction by intentional inclusion of the tumor-forming Ti plasmid in their hybrids were headed in the opposite direction from that required to produce hybrid bacteria capable of entering into successful endosymbiotic relationships with plant hosts.

Other unsuccessful attempts to extend the range of nitrogen-fixing Rhizobium species include the insertion of the Ti plasmid from Agrobacterium tumefaciens into selected Rhizobium strains. See P.J.J. Hooykass, et al., J. Gen. Microbiol., 98:477-484 (1977), which is specifically incorporated herein by reference in its entirety. These studies resulted in Rhizobium strains able to produce galls

on non-leguminous dicotyledonous plants which will fix nitrogen but only under anaerobic conditions not naturally occurring in such plants. The criticisms outlined in the previous paragraph also applies to this work.

Despite substantial economic incentive to do so, the prior art has been unable to prepare hybrid nitrogen-fixing bacteria capable of entering into endosymbiotic relationships with non-leguminous plant hosts or any other hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with plant hosts.

SUMMARY OF THE INVENTION

A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host has been discovered which comprises combining genetic material of an agricultural-chemical-producing microorganism and a plant-infecting microorganism which infects the plant host (hereinafter sometimes referred to as an "infecting microorganism") to form hybrid microorganisms and selecting from the hybrid microorganisms those hybrid microorganisms which are capable of producing an agricultural chemical and of entering into an endosymbiotic relationship with the plant host and which do not create manifestations of disease in the plant host.

The hybrid microorganisms are formed by combining some or all of the genetic material of the agricultural-chemical-producing microorganism and the infecting microorganism. The genetic material is combined through use of various techniques, such as recombinant DNA, recombinant RNA, cell fusion, conjugation, plasmid transfer, transformation, transfection, transduction, and microinjection.

The microorganisms resulting from the process of the present invention may be introduced into crop plants by a variety of means, including injection, and may be employed to coat agricultural seed, to infect agricultural seed, in the preparation of a soil drench, and the like. The hybrid microorganisms of the present invention, upon association

with crop plants, are capable of supplying some or all of the plants' agricultural chemical needs. Agricultural chemicals that can be produced by hybrid bacteria in accordance with the present invention include fertilizers, particularly fixed-nitrogen fertilizers; antibiotics, including antibacterial, antiviral, antifungal, insecticidal, nematocidal, miticidal, and herbicidal agents; plant growth regulators, including plant hormones, and the like.

In a preferred embodiment, there has been discovered a method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host comprising:

(A) in any convenient order;
  (1) identifying a plant host;
  (2) identifying an infecting microorganism which infects the plant host;
  (3) selecting mutants of the infecting microorganism with one or more selectable traits in addition to ability to infect the host; and
  (4) selecting an agricultural-chemical-producing microorganism having one or more selectable traits;

(B) forming fusion hybrids of the infecting microorganism and the agricultural-chemical-producing microorganism;

(C) selecting serially from the products of the previously performed step or substep and in any convenient order:
  (1) a subgroup comprising those hybrids having both at least one of the selectable traits of the agricultural-chemical-producing microorganism and at least one of the selectable traits of the infecting microorganisms;
  (2) a subgroup comprising those hybrids which manifest the ability to interact with plant

tissue in the manner in which the infecting microorganism interacts with plant tissue during the initial phase of infection in the plant host;

(3) a subgroup comprising those hybrids which, upon application to the host, do not create manifestations of a disease; and

(4) a subgroup comprising those hybrids having the ability to produce the agricultural chemical if not previously selected for; and

(D) selecting hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host by selecting from the products of the last performed step of stages (C)(1) to (C)(4) those hybrid microorganisms capable of improving the performance of the plant host under conditions wherein its performance could be improved by direct application of the agricultural chemical or the agricultural-chemical-producing microorganism to the plant.

In a particularly preferred embodiment, the hybrid microorganisms are hybrid bacteria produced by protoplast or spheroplast fusion of an infecting bacterium and an agricultural-chemical-producing bacterium having the same response to gram stain. Certain gram-positive bacteria, notably members of the genus Bacillus, Streptomyces, and Clavibacter are particularly useful for the formation of the preferred hybrid bacteria.

In another preferred embodiment, the hybrid agricultural-chemical-producing microorganisms of the present invention capable of entering into endosymbiotic relationships with a plant host are formed by the steps comprising:

(A) in any convenient order:

(1) identifying the plant host; and

(2) identifying an infecting microorganism which infects the plant host;

(B) preparing a vector capable of being transferred into and replicating in the infecting microorganism;

(C) preparing an expression module capable of directing the production of an agricultural chemical by the infecting microorganism;

(D) placing the expression module in the vector to create an expression vector capable of being transferred into and replicating in the infecting microorganism, the expression vector being capable of directing the production of the agricultural chemical by the infecting microorganism;

(E) transforming the infecting microorganism with the expression vector to produce hybrid microorganisms;

(F) selecting for the hybrid microorganisms;

(G) selecting serially from the selected hybrid microorganisms and in any convenient order:

(1) a subgroup comprising those hybrid microorganisms which manifest the ability to interact with plant tissue in the manner in which the infecting microorganism interacts with plant tissue during the initial phase of infection in the plant host;

(2) a subgroup comprising those hybrid microorganisms which, upon application to the host, do not create manifestations of disease; and

(3) a subgroup comprising those hybrid microorganisms having the ability to produce the agricultural chemical if not previously selected for; and

(H) selecting hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host by selecting from the products of the last performed step of steps (G)(1) to (G)(3) those hybrid microorganisms capable of improving the performance

of the plant host under conditions wherein the performance would be improved by direct application of the agricultural chemical or the agricultural-chemical-producing microorganism to the plant host.

In a particularly preferred embodiment, the hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host are formed by the steps comprising:

(A) in any convenient order:
  (1) identifying a plant host; and
  (2) identifying an infecting microorganism which infects the plant host;
(B) preparing an integration vector containing an integration sequence and capable of integrating into the genome of the infecting microorganism;
(C) preparing an expression module capable of directing the production of an agricultural chemical by the infecting microorganism;
(D) placing the expression module within the integration sequence of the integration vector, thereby producing a modified integration vector capable of integrating into the genome of the infecting microorganism and directing the production of the agricultural chemical by the infecting microorganism;
(E) transforming the infecting microorganism with the modified integration vector to produce hybrid microorganisms;
(F) selecting for the hybrid microorganisms;
(G) selecting serially from the selected hybrid microorganisms and in any convenient order:
  (1) a subgroup comprising those hybrid microorganisms which manifest the ability to interact with plant tissue in the manner in which the infecting microorganism interacts with plant tissue

during the initial phase of infection in the plant host;

(2) a subgroup comprising those hybrid microorganisms which, upon application to the host, do not create manifestations of disease; and

(3) a subgroup comprising those hybrid microorganisms having the ability to produce the agricultural chemical, if not previously selected for; and

(H) selecting hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host by selecting from the products of the last performed step of steps (G)(1) to (G)(3) those hybrid microorganisms capable of improving the performance of the plant host under conditions wherein the performance would be improved by direct application of the agricultural chemical or the agricultural-chemical-producing microorganism to the plant host.

The processes are capable of producing agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with both monocotyledonous and dicotyledonous plants. For dicotyledonous plants, infecting bacteria of the genus Agrobacterium are preferred, with strains of Agrobacterium tumefaciens being particularly preferred. Species of the genus Erwinia, such as Erwinia carotovora may also be used, as may species of the genus Pseudomonas, such as Pseudomonas solanacearum and Pseudomonas syringae, of the genus Xanthomonas, such as Xanthomonas campestris, and of the genus Streptomyces, such as S. ipomoea. For monocotyledonous plants, species of the genus Erwinia, such as Erwinia stewartii, are preferred infecting bacteria. Species of the genus Xanthomonas, such as Xanthomonas campestris, species of the genus Azospirillum,

such as Azospirillum lipoferum and Azospirillum brasilense, and species of the genus Pseudomonas, such as Pseudomonas syringae, are also contemplated as being useful. Pseudomonas syringae is contemplated as being particularly useful as an infecting bacterium for the formation of fusion products applicable to cereals, including temperate cereals and rice. Clavibacter species such as C. xyli subsp. xyli and C. xyli subsp. cynodontis are particularly useful for grasses, such as maize, sorghum, and the like.

Preferred agricultural-chemical-producing microorganisms and the chemicals and/or applications for which their metabolic products are useful are identified in Table I below and include organisms having the ability to produce fertilizers, including fixed nitrogen and chemicals capable of solubilizing phosphates, antibiotics, including antibacterial compounds, antifungal compounds, antiviral compounds, insecticides, nematocides, miticides and herbicides, and plant growth regulators. Additionally, useful organisms may be selected or modified to produce other agricultural chemicals, as above defined, including fragrances, antifeeding agents and the like.

The processes of the present invention result in novel, stable hybrid microorganisms having the ability to produce one or more agricultural chemicals and enter into endosymbiotic relationships with a plant host. The nitrogen-fixing hybrid bacteria of the present invention, for example, have been shown to improve the yield of non-leguminous crop plants growing under low fixed nitrogen conditions by amounts of from 10 to 180%, due primarily, it is believed, to fixed nitrogen produced by the hybrids of nitrogen-fixing bacteria and infecting bacteria in the course of their endosymbiotic relationship with the plant host.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned from the practice of the invention. The objects and advantages may be realized and attained by means of the

instrumentalities and combinations particularly pointed out in the appended claims.

The hybrid agricultural-chemical-producing microorganisms produced by the above-described method may be further modified by natural or artificial genetic techniques to improve their performance as sources of agricultural-chemical for the plant host. Such modification could result, for example, in the ability to excrete the agricultural chemical, such as fixed nitrogen, including the ability to excrete the agricultural chemical in a particular form, such as fixed nitrogen in the form of amino acids; the ability to continue production of the agricultural chemical even in the presence of adequate amounts of that chemical from other sources; in a reduction of the hybrid's resistance to cold temperature (i.e., to prevent unintended proliferation of the hybrids from year to year); in enhancement of the hybrid's ability to withstand drought, disease, or other physiological stress; in the introduction of additional agricultural-chemical-producing functions; or in modification of the hybrid so that it cannot grow outside the plant host.

While the steps of the above-described method may be carried out in any convenient order, it is desirable that the process of selection for agricultural-chemical-producing ability and for ability to interact with plant tissue in the manner in which the infecting microorganism interacts with plant tissue during the initial phases of infection in the host plant be carried out two or more times before the step relating to selection of those hybrids which do not manifest symptoms of disease in the plant host.

The selectable traits associated with the infecting bacterium may be antibiotic resistance, need for specific nutritional supplementation (auxotrophism), resistance to toxins, or the like. The selectable traits associated with the agricultural-chemical-producing microorganism may be the ability to produce an agricultural chemical alone or in combination with one or more of those traits previously mentioned with respect to the infecting microorganism. The

interaction with plant tissue screened for in the above-described method may be, for example, the ability to bind to plant tissue, the ability to spread throughout the vascular system of the plant, or the like. Where the interaction associated with the initial phase of infection is binding to plant cells, it is desirable to limit further the microbial subgroup resulting from the penultimate step by selection of a microbial subgroup for further screening in accordance with the above-described process comprising those hybrids which spread most readily throughout the plant host.

By appropriate selection of the infecting microorganism, hybrids may be obtained which are capable of entering into endosymbiotic relationships with cereals, such as wheat, triticale, barley, rye, rice and oats; grasses, such as brome grass, blue grass, tall fescue grass, fine fescue grass, ryegrass, and Bermuda grass; tropical grasses, such as sugar cane, corn, millet and sorghum; solanaceous plants, such as potatoes, tomatoes, tobacco, eggplant and pepper; brassicaceous plants such as cauliflower, broccoli, cabbage, kale and kohlrabi; other vegetables, such as carrot and parsley; other agriculturally grown plants, such as sugar beets, cotton, fruit trees, berry plants, and grapes; and economically important tree species, such as pine, spruce, fir and aspen. The process of the present invention and the resulting microorganisms may also be used to fulfill some or all of the fixed-nitrogen or other agricultural chemical requirements of leguminous plants, such as soybeans, alfalfa, clover, field beans, mung beans, peas and other pulses, as a supplement, for example, to fixed nitrogen provided by species of Rhizobium associated with nodules on their roots.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one embodiment of the invention and, together with the description, serve to explain the principles of the invention.

Figure 1 depicts a partial restriction map of the plasmid pCG300.

Figure 2 depicts a partial restriction map of the plasmid pCG306, which is derived from pCG300.

Figure 3 depicts a partial restriction map of the plasmid pCG6.

Figure 4 depicts a truncated B. thuringiensis delta endotoxin gene fused to a kanamycin resistance gene in the vector m8TK65.

Figure 5 depicts the insertion of a truncated B. thuringiensis delta endotoxin gene fused to a kanamycin resistance gene into pCG6 Neo^S and the insertion of an expression module containing these genes into pCG300.

Figure 6 show an abbreviated restriction map of the delta-endotoxin gene of B. thuringeinsis subsp. kurstaki HD73 and flanking sequences.

Figure 7 shows the sequence of 4.993 HincII Fragment of Tn916.

Figure 8 shows the restriction map of Tn916.

Figure 9 shows a Tn916 restriction map with insertions of Tn5 and the resulting effects on behavior.

Figure 10 shows the construction of pCG563.

Figure 11 shows the modification of the delta-endotoxin gene of B. thuringeinsis subsp. kurstaki HD73.

Figure 12 shows the altered and native sequences of the delta-endotoxin gene of B. thuringeinsis subsp. kurstaki HD73 and flanking sequences.

Figure 13 shows the construction of the Bt integration plasmid pCG741.

Figure 14 shows the restriction maps of four probes, the Bt probe, the TetM probe, the pGEM probe, and the Cxc 209 probe.

Figure 15 shows the restriction enzyme sites used for southern analysis of pCG741 in MDR1.586.

Figure 16 shows the results of a Southern hybridization analysis of DNA.

Figure 17 shows the results of a Southern hybridization analysis of DNA samples.

Figure 18 shows the population dynamics of a Cxc/Bt construction in greenhouse corn.

Figure 19 shows a standard curve generated by scanning densitometry of immunovisualized Western blot.

Figure 20 shows a densitometry of Western blots.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Reference will now be made in detail to the presently preferred embodiments of the invention, which together with the following examples, serve to explain the principles of the invention.

As noted above, the present invention relates to a method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host which comprises combining genetic material from an agricultural-chemical-producing microorganism and an infecting microorganism which infects the plant host to form hybrid microorganisms and selecting from the hybrid microorganisms those capable of producing the agricultural chemical, which do not create manifestations of a disease in the plant host, yet which are capable of entering into an endosymbictic relationship with the plant host. Preferably, the hybrid agricultural-chemical-producing microorganisms are capable of improving the performance of the plant host under conditions wherein the performance would be improved by direct application of the agricultural chemical or the agricultural chemical-producing microorganism to the plant. Most preferably, the agricultural-chemical-producing microorganism and the infecting microorganism are bacteria. As used throughout this specification, the term "direct application" means application to the whole plant or any part of the plant, including systemic or partial systemic application.

The term "microorganism" as used herein is intended to encompass bacteria, fungi (including yeast), and algae. The term "bacteria" as used herein is intended to encompass

bacteria, bacteria-like organisms and their equivalents, including gram positive bacteria, gram negative bacteria, actinomycetes and the like. The only limitations on the taxonomic classification of the microorganisms used in accordance with the present invention is that some or all of their genetic material be capable of being used to produce viable hybrid organisms expressing phenotypical properties of both parents as more fully described herein.

"Infecting microorganisms" as used throughout this specification is intended to connote not only microorganisms which enter and live within the plant and normally produce symptoms of disease but also microorganisms which enter and live within the plant symbiotically or commensally. Indeed, some of the plant infecting microorganisms used in accordance with the present invention, while normally creating pathogenic responses in some plant hosts, will not normally produce such responses in all plant hosts.

Performance can be determined and evaluated by those skilled in the art by considering any one or more of a multitude of factors. These include: 1) resistance to environmental stress, such as drought, high salinity, pests, and harmful chemicals; 2) increased yield; 3) faster maturity; 4) less dependence on added nutrients; and 5) enhanced quality of the plant product of interest.

The hybrid microorganisms are formed by combining some or all of the genetic material of the agricultural-chemical-producing microorganism and the infecting microorganism. The genetic material is combined by the techniques of recombinant DNA, recombinant RNA, cell fusion, conjugation and plasmid transfer, transformation, transfection, transduction, and microinjection. Some of these techniques are described in Maniatis, T., E.F. Fritsch, and J. Sambrook, Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory 1982) and Miller, J.H., Experiments in Molecular Genetics (Cold Spring Harbor Laboratory 1972), both of which are specifically incorporated herein by reference in their entirety. Selection of a technique by which the hybrid

microorganisms of the present invention may be formed will generally be within the capabilities of one of ordinary skill in the art based on the above-referenced scientific literature and the teachings disclosed herein.

In a preferred embodiment, the agricultural-chemical-producing microorganisms of the present invention capable of entering into endosymbiotic relationships with a plant host may be formed by steps comprising:

(A) in any convenient order:
  (1) identifying the plant host;
  (2) identifying an infecting microorganism which infects the plant host;
  (3) selecting mutants of the infecting microorganism with one or more selectable traits in addition to ability to infect the host; and
  (4) selecting an agricultural-chemical-producing microorganism having one or more selectable traits;

(B) forming fusion hybrids of the infecting microorganism and the agricultural-chemical-producing microorganism;

(C) selecting serially from products of the previously performed step or substep and in any convenient order:
  (1) a subgroup comprising those hybrids having both at least one of the selectable traits of the agricultural-chemical-producing microorganism and at least one of the selectable traits of the infecting microorganism;
  (2) a subgroup comprising those hybrids which manifest the ability to interact with plant tissue in the manner in which the infecting microorganism interacts with plant tissue during the initial phase of infection in the plant host;

(3) a subgroup comprising those hybrids which, upon application to the host, do not create manifestations of a disease; and

(4) a subgroup comprising those hybrids having the ability to produce the agricultural chemical if not previously selected for; and

(D) selecting hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host by selecting from the products of the last performed step of steps (C)(1) to (C)(4) those hybrid microorganisms capable of improving the performance of the plant host under conditions wherein the performance could be improved by direct application of the agricultural chemical or the agricultural-chemical-producing microorganism to the plant.

As used in the steps above, the term "selecting" means any intervention or combination of interventions such that the desired microorganism can be recognized either by its ability to survive or by its unique properties.

As indicated, the steps of the method may be performed in any convenient order. Preferably, they are performed in the order recited with the selectable traits of the agricultural-chemical-producing microorganism screened for in step (C)(1) including the ability of the hybrid to produce the agricultural chemical in question, as in the case of fixed-nitrogen producing bacteria. If screening for ability to produce the agricultural chemical in question is not conducted in the first screening step, then it is preferred to conduct such screening (step C(4)) prior to or concurrently with the screening in the plant host contemplated by steps (C)(2) and (C)(3). When performed in the order recited, the antibiotic resistance markers or the like constituting the selectable traits associated with the infecting bacterium need not survive subsequent screening procedures.

The stable hybrid microorganisms resulting from processes of the present invention are distinguished from hitherto known organisms in that they have both the ability to produce agricultural chemicals and the ability to enter into endosymbiotic relationships with a plant host. The endosymbiotic relationship referred to is one in which the organism actually exists within and spreads throughout all or a portion of the plant host, without causing a pathogenic response, deriving some or all of its energy requirements from carbohydrates and other materials produced by the plant host and providing agricultural chemicals which may be used by the plant host to supplement those otherwise available.

The plant hosts with which the microorganisms of the present invention may establish endosymbiotic relationships may include virtually all economically important crop plants. In addition, any particular hybrid microorganism is not necessarily limited to one particular species of plant host. Its host range will depend upon many factors, including the infecting microorganism from which it is derived. At the same time, the phenotypic traits controlled by the genetic material of the infecting microorganism from which it is derived will place limits on its host range, as will other traits specifically engineered into the hybrid organism.

The process of the present invention has been shown to be capable of producing stable hybrid microorganisms having the ability to produce an agricultural chemical and of entering into endosymbiotic relationships with both monocotyledonous and dicotyledonous plant hosts. A particularly important group of plant hosts for which the products of the present invention may serve as endosymbiotic agricultural-chemical-producing microorganisms are the cereals, including temperate cereals, such as wheat, triticale, barley, rye, rice and oats. Endosymbiotic agricultural-chemical-producing microorganisms formed in accordance with the present invention may also be useful in supplementing the agricultural chemical, including fixed nitrogen, needs of economically important sod and forage

grasses, such as brome grass, blue grass, tall fescue grass and Bermuda grass. Similarly, the organisms of the present invention have a demonstrated ability to increase the yields of tropical grasses, such as sugar cane, corn, millet and sorghum. Solanaceous plants, such as potatoes, tomatoes, tobacco, eggplant and pepper are suitable plant hosts to which the organisms of the present invention may be applied, as are brassicaceous plants, such as cauliflower, broccoli, cabbage, kale and kohlrabi. Miscellaneous vegetables such as carrots and parsley; other agriculturally grown plants, such as sugar beets, cotton, fruit trees, berry plants and grapes; and economically important tree species, such as pine, spruce, fir and aspen, may also serve as plant hosts for the organisms of the present invention.

Even though plants may have symbiotic relationships with microorganisms which produce agricultural chemicals, such as leguminous plants with bacteria of the genus Rhizobium which fix atmospheric nitrogen anaerobically in root nodules, these plants are obtained in the greatest yield when provided with supplemental agricultural chemical sources through fertilization or otherwise. Accordingly, it is envisioned that such supplemental agricultural chemicals, including supplemental fixed nitrogen, for such plants may be provided by microorganisms formed in accordance with the present invention capable of producing agricultural chemicals, including fixed nitrogen, and capable of entering into endosymbiotic relationships with these leguminous plants.

The agricultural-chemical-producing microorganism employed in the present invention may be any microorganism that produces an agricultural chemical or chemical effect of interest. Agricultural-chemical-producing microorganisms that may be employed in the present invention are those capable of producing antibiotics, antifungal agents, antiviral agents, insecticides, nematocides, miticides, herbicides, plant growth regulating compounds, fertilizing chemicals other than fixed nitrogen, fragrances, sensory enhancing chemicals, antifeeding agents and the like.

Selection of such agricultural-chemical-producing microorganism will be within the capabilities of those skilled in the art in light of the teachings and illustrations contained herein.

In one embodiment, the agricultural-chemical-producing microorganism is a bacterium capable of fixing atmospheric nitrogen aerobically. Such a bacterium may be selected from the genera Azotobacter, Azomonas, Beijerinckia, and Derxia, among others. A preferred group of nitrogen fixing bacteria are those from the genus Azotobacter, such as Azotobacter vinelandii, Azotobacter paspali, Azotobacter beijerinckia and Azotobacter chroococuum. Azotobacter vinelandii is particularly preferred because its genetics and regulation of nitrogen fixation have been extensively studied and because it has a demonstrated ability to accept and express genetic material from other bacterial genera. See G.P. Roberts, et al., "Genetics and Regulation of Nitrog.n Fixation," Ann. Rev. Microbiol., 35:207-35 (1981). Although Azotobacter and other significant aerobic nitrogen-fixing bacteria are generally gram-negative bacteria, it is to be understood that the techniques of the present invention are applicable to both gram-positive and gram-negative bacteria.

A table of exemplary agricultural-chemical-producing bacteria contemplated as useful in accordance with the present invention and the agricultural chemical or chemical effects they produce appear in Table I.

Table I. Agricultural-Chemical-Producing Microorganisms

Group	Genus	Species	Chemical Application
Antibiotics Anti-fungal Agents	Streptomyces	cacaoi var. asoensis	produces polyoxins which prevent rice sheath blight
		griseus	produces cycloheximide which prevents

		onion downy mildew
	griseo-chromagenes	produces blasticidin S which prevents rice blast
	hygrosco-picus var. limoneus	produces validamycin A which prevents rice sheath blight
	kasugaensis	produces kasugamycin which prevents rice blast
	kitazawaensis	produces ezomycin which prevents stem rot of kidney beans
	Tendae Tu/901	produces nikkomycin which is active against various fungi
Pseudomonas	fluorescens strain B10	causes iron to become unavailable to other microorganisms such as fungi
Streptoverticillium	rimofaciens	produces mildiomycin which is active against various

			powdery mildews
Antibacterial Agents	Streptomyces	chibaensis	produces cellocidin which prevents rice bacterial leaf blight
		griseus	produces streptomycin which prevents various bacterial diseases
		lavendulae No. 6600 Gc-1	produces laurus in which prevents bacterial leaf blight of rice plants
		spheroides	produces novobiocin which prevents tomato canker
		venezuelae	produces chloramphenicol which presents bacterial leaf blight of rice plants
		viridifaciens	produces tetracycline which prevents various bacterial diseases

	Agrobacterium	radio-bacteria strain 84	produces agrocin - 84
	Nocardia	sp.	produces myomycin
Antiviral Agents	Streptomyces	hygroscopicus var. aabomyceticus	produces aabomycin A which inhibits its TMV multiplication in tobacco tissue
		lavendulae No. 6600 GC-1	produces laurusin which inhibits TMV multiplication in tobacco tissue
		miharuensis	produces miharamycin which is effective in controlling TMV, CMV, PVX, and RSV
Insecticides	Bacillus	cereus	affects hymenoptera lepidoptera, coleoptera
		euloomarahae	effects coleopteran larvae, especially Japanese beetle

	fribou-gensis	affects coleopteran larvae, especially Japanese beetle
	lentimorbus	affects coleopteran larvae, especially Japanese beetle
	moritai	affects seed corn maggot
	popilliae	affects coleopteran larvae, especially Japanese beetle
	thurin-giensis var. alesti, dendro-limus, entomo-cidus, kurstaki, sotto, tenebrionis, israelensis, berliner	affect numer-ous insect pest larvae
Clostridium	bravi-daciens	affects tent caterpillar
	malacusomae	affects tent caterpillar

	Streptomyces	strain B-41-146	produces milbemycin
		avermitilis	produces avermactins
Nematocide	Bacillus	penetrans	effective against root-knot nematodes
	Streptomyces	avermitilis	produces avermectins
Miticide	Steptomyces	aureus S-3466	produces tetranactin
		avermitilis	produces avermectins
Herbicides	Streptomyces	griseus	produces cycloheximide
		saganoensis	produces herbicidin A, B
		sp.	produces bialaphos
		sp.	produces anisymycin
	Pseudomonas	syringae pv. atropurpurae	produces coronatine which causes chocolate spot disease on Italian ryegrass
Fertilizers Nitrogen-fixing	Azotobacter	beijerinckia, enroococcum, paspali, vinelandii	fix atmospheric nitrogen
	Azomonas		fix atmospheric nitrogen
	Beijerinckia		fix atmospheric nitrogen

	Derxia		fix atmospheric nitrogen
Phosphate Solubilizing	Bacillus	circulans, floure-scens, magaterium, mesen-tericus, mycoides, polymyxa, pulvi-taciens, subtilis	solubilize bound phos-phate
	Pseudomonas	calcis, liqui-faciens, putida, rathonia, striata	solubilize bound phos-phates
Plant Growth Reg-ulators	Azotobacter	sp.	produce vari-ous vitamins auxins, cyto-kinins, giberellin-like substance and other plant hormones
	Agrobacterium	sp.	
	Pseudomonas	sp.	
	Bacillus	megaterium, subtilis	

The infecting microorganism capable of infecting the plant host employed in the present invention may be any of a wide variety of bacterial species which infect the plant host under consideration. Preferably, the infecting microorganism should have a known method of interaction with the plant host during the initial phase of infection. The infecting

microorganism may be either a pathogen, including latent pathogens, or an endosymbiotic species. In the case of pathogens, it is preferred that the pathogen create a visible manifestation of the disease associated with it. Exemplary pathogens include species of the genera Agrobacterium and Erwinia. Exemplary endosymbiotic species include species of Azospirillum, Corynebacterium, and Clavibacter. The species of Azospirillum are known to live in the roots of tropical grasses, such as sugar cane, and temperate grasses, such as wheat, without causing manifestations of disease. Certain species of Corynebacterium live in wheat and corn. It has been discovered that a species of Clavibacter lives in corn.

Most preferably, the infecting microorganism is a strain which is specific, or nearly specific, to the particular crop plant host under consideration in order to limit the potential for spread of the hybrid microorganisms of the present invention to plants other than those with which they are intended to enter an endosymbiotic relationship. Many plant infecting microorganisms and their methods of interaction with plant hosts are described in the scientific literature. Particular reference in this regard is made to M.S. Mount et al., Phytopathogenic Prokaryotes, Volumes I and II (1982), which is specifically incorporated herein by reference. In addition, xylem-limited bacteria, such as the bacterium that causes Pierce's disease and that which inhabits C-4 grasses such as Bermuda grass, will be useful in the practice of this invention. Such bacteria are described in Wells, et al., Phytopathology 71:1156-1161 (1981) and Raju, et al., Am. J. Enol. Vitic. 31:17-21 (1980), which are incorporated herein by reference.

Many pathogenic microorganisms are known to form strains essentially undifferentiable from each other except for their preference for particular plant hosts. Such host-specific pathogen strains are known as pathovars for the plant host involved. It is envisioned that the present invention will be particularly useful with pathovars for the particular host under consideration, including pathovars of the genera

Agrobacterium, particularly Agrobacterium tumefaciens; Erwinia, particularly Erwinia stewartii and Erwinia carotovora; Pseudomonas, particularly Pseudomonas solanacearum and Pseudomonas syringae; Xanthomonas, particularly Xanthomonas campestris; and similar bacteria.

Particularly preferred non-pathogenic endosymbionts include species of the genus Azospirillum, particularly Azospirillum lipoferum and Azospirillum brasilense, the genus Acremonium, particularly Acremonium typhinum and Acremonium coenophialum, and the genus Balansia. Infecting microorganisms envisioned as suitable for use in accordance with the present invention include those listed in Table II.

Table II. Plant-Infecting Microorganisms

Type	Genus	Species
Gram-negative bacteria aerobic rods and cocci	Pseudomonas	agarici, andropogonis, avenae, caricapapayae, caryophylli, cichorii, cissicola, gladioli, glumae, marginalis, pseudoalcaligenes subsp. citrulli, rubrilineans, rubrisubalbicans, solanacearum, syringae, tolaasii, viridiflava
	Xanthomonas	albilineans, ampelina, axonopodis, campestris, fragariae
	Agrobacterium	rhizogenes, rubi, tumefaciens
Facultatively anaerobic rods	Erwinia	amylovora, ananas, cancerogena, carnegieana, carotovora subsp. atroseptica, carotovora subsp.

		carotovora, chrysanthemi, cypripedii, dissolvens, herbicola, mallotivora, milletiae, nigrifluens, nimipressuralis, quercina, rhapontici, rubrifaciens, salicis, stewartii, tracheiphila, uredovora
Gram-negative bacteria	Azospirillum	lipoferum, brasilense
Gram-positive bacteria Actinomycetes and related organisms	Coryne-bacterium	betae, beticola, fascians, flaccumfaciens, ilicis, insidiosum, michiganense, nebraskense, oortii, poinsettiae, sepedonicum
	Clavibacter	xyli
	Streptomyces	ipomoea, scabies
Fungi	Acremonium	typhinum, coenophialum

Pathovars of Agrobacterium tumefaciens are particularly preferred for formation of stable hybrids capable of producing agricultural chemicals and of entering into endosymbiotic relationships with dicotyledonous plant hosts. Agrobacterium tumefaciens has a well identified interaction with the plant during the initial phases of infection in that it binds to plant cell tissue. The ability of Agrobacterium tumefaciens to bind to plant cell tissue in vitro has been

demonstrated and has been shown to parallel the host range for tumor formation by Agrobacterium tumefaciens in vivo. See A.G. Matthysse, et al., "Plant Cell Range for Attachment of Agrobacterium tumefaciens to Tissue Culture Cells," Physiological Plant Pathology, 21:381-387 (1982); A.G. Matthysse, et al., "Binding of Agrobacterium tumefaciens to Carrot Protoplasts," Physiological Plant Pathology, 20:27-33 (1982), both of which are specifically incorporated herein by reference.

Agrobacterium tumefaciens is the preferred infecting microorganism for use in forming stable hybrids capable of producing agricultural chemicals and of entering into endosymbiotic relationships with solanaceous plants, such as potatoes, tomatoes, tobacco, eggplant and pepper; brassicaceous plants, such as cauliflower, broccoli, cabbage, kale and kohlrabi; vegetables, such as carrot and parsley and other agriculturally grown plants, such as sugar beets, cotton, fruit trees, berry plants and grapes. Other infecting microorganisms suitable for use in accordance with the present invention to produce agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with dicotyledonous plants include Erwinia carotovora, Pseudomonas solanacearum, Pseudomonas syringae and Xanthomonas campestris.

Infecting microorganisms such as Pseudomonas syringae and Xanthomonas campestris are envisioned as being particularly useful in the formation of stable hybrid microorganisms capable of producing agricultural chemicals and of entering into endosymbiotic relationships with monocotyledonous cereal crops, such as wheat, barley, rye, rice and oats, and also with grasses, such as brome grass, blue grass, tall fescue grass and Bermuda grass.

Corynebacterium and Clavibacter species are useful as the infecting microorganism for such monocots as grasses, wheat, corn, and sorghum.

Strains of Erwinia stewartii and Clavibacter xyli subsp. cynodontis or subsp. xyli are particularly preferred as the

infecting microorganism useful in forming stable hybrid microorganisms capable of producing agricultural chemicals and of entering into endosymbiotic relationships with monocotyledonous plants, such as tropical grasses, such as sugar cane, corn, millet and sorghum, and small grains such as rice. Strains of Pseudomonas syringae and Xanthomonas campestris are also envisioned as useful in these applications.

The above-identified strains of Azospirillum are envisioned as being useful in monocotyledonous plants, including tropical and temperate grasses, such as sugar cane and wheat, among others.

Species of the genus Balansia and the genus Acremonium, particularly Acremonium typhinum and Acremonium coenophialum, are useful as the infecting microorganism for grasses and forages, such as tall fescue, fine fescue, and ryegrass.

Streptomyces species are useful as the infecting microorganism of root and tuber crops, such as sweet potato, white potato, sugar and other beets, and radishes.

It is to be understood that the selection of a particular plant-infecting microorganism and a particular agricultural-chemical-producing microorganism for use in accordance with the present invention will be within the capabilities of one having ordinary skill in the art through the exercise of routine experimentation in light of the teachings contained herein and inferences logically to be drawn from the disclosure of the present invention. The characteristics of microorganisms contemplated as useful as either the agricultural-chemical-producing microorganism or the infecting microorganism in processes of the present invention may be determined by routine experimentation or by reference to standard compendia, such as Bergey's Manual of Determinative Bacteriology, and the like.

For use in the present invention, it is preferred that mutants of the desired infecting microorganism be selected which have one or more selectable traits in addition to the ability to infect the plant host under consideration.

Specifically, mutants of the infecting microorganism should be selected which have traits which are selectable in vitro. Such traits include antibiotic resistance, the need for specific nutritional supplementation (auxotrophism), resistance to toxins such as heavy metals, combinations thereof, and the like, which will allow rapid in vitro screening of the hybrid microorganisms to identify those containing genetic material from the mutant infecting microorganism. In the absence of a selectable trait, physical means can be used to kill one or both parents but which will allow hybrids to survive. Such physical means include ultraviolet light and heat.

Antibiotic resistance is the preferred selectable trait, and it is particularly preferred that the selected infecting mutant microorganism have resistance to at least two antibiotics. Dual antibiotic resistance, or redundancy in other selectable traits, ensure that the initial screening of the hybrids will identify only those having genetic material from both the agricultural-chemical-producing microorganism and the selected infecting mutant microorganism and reduces the chances of the survival or formation of mutant antibiotic resistant strains of the agricultural-chemical-producing microorganism. This is of particular value with fusion hybrids and is of little or no significance if cloned genes are employed. For example, in formation of agricultural-chemical-producing hybrids capable of producing fixed nitrogen and of entering into endosymbiotic relationships with monocotyledonous plants, strains of Erwinia stewartii which have resistance to both streptomycin and tetracycline are preferred. In the formation of hybrids capable of producing fixed nitrogen and of entering into endosymbiotic relationships with dicotyledonous plants, strains of Agrobacterium tumefaciens which are both streptomycin resistant and kana/mycin resistant are preferred. Such mutant strains of plant infecting microorganisms are available from microorganism depositories such as the American Type Culture Collection in Rockville, Maryland, or

may be generated from the wild type by techniques well known in the art.

Where hybrids of the agricultural-chemical-producing microorganism are not to be screened initially for the ability to produce the agricultural chemical in question, the agricultural-chemical-producing microorganism may be a mutant with one or more additional selectable traits, preferably in vitro selectable traits, such as those mentioned above with respect to the infecting microorganism. Thus, for example, if the agricultural chemicals of interest is an insecticide, nematocide or the like, the agricultural-chemical-producing microorganism may be a mutant which has selectable antibiotic resistance or sensitivity or a selectable need for specific nutritional supplementation. Obviously, these traits should not be identical to the selectable traits of the infecting microorganism since the initial screen would otherwise be unable to eliminate non-hybrid organisms. While the presence in the hybrids of such additional selectable traits from the agricultural-chemical-producing microorganism does not guarantee the presence of agricultural-chemical-producing capability in the products of the initial screening procedure, it sufficiently enriches the population with true hybrid organisms to the point where there is a reasonable predictability of success in identifying those hybrid organisms with the agricultural-chemical-producing capability in subsequent screening.

In a particularly preferred embodiment, the hybrids of the present invention are formed by protoplast or spheroplast fusion of bacteria following the outline of techniques generally employed in the prior art. Known protoplast and spheroplast fusion techniques are described in D.A. Hopwood, "Genetic Studies With Bacterial Protoplasts," Ann. Rev. Microbiol, 35:237-72 (1981), and in R.L. Weiss, J. Bacteriol., 128:668-70 (1976), both of which are specifically incorporated herein by reference in their entirety. In this embodiment, it is preferred, although not necessary, that the

infecting bacterium and the agricultural-chemical-producing bacterium employed exhibit the same response to gram stain.

In general, the fusion procedure involves the removal of the cell wall from both the agricultural-chemical-producing bacteria and the infecting bacteria, fusion of the infecting bacteria and agricultural-chemical-producing bacteria cells in a fusion-inducing medium, such as polyethylene glycol, and regeneration of the cell wall about the fusion hybrids containing genetic material from both the infecting bacterium and the agricultural-chemical-producing bacterium. Fusion and regeneration of the cell wall are conducted at low temperatures so that rates of expressible genetic recombination are favored in relation to rates of enzymatic destruction of genetic material in the newly formed hybrids.

Following initial formation of fusion hybrids, the genetic make-up of the bacterial population is relatively unstable for a period of two or three days, during which much genetic recombination apparently occurs. During this time period, those stable fusion hybrid capable both of manifesting the one or more selectable traits associated with the agricultural-chemical-producing bacteria and of manifesting the one or more selectable traits associated with the infecting bacterium are selected. It is particularly preferred that the agricultural-chemical-producing trait also be selected during this step. For example, fusion products of Azotobacter vinelandii and Erwinia stewartii formed from strains of Erwinia stewartii which are streptomycin and tetracycline resistant are grown in a nitrogen-free medium containing both streptomycin and tetracycline. Thus, only those organisms containing both the genetic material associated with nitrogen fixation from Azotobacter vinelandii and the genetic material associated with streptomycin and tetracycline resistance from Erwinia stewartii will survive the two to three day incubation period.

The surviving fusion hybrids manifesting both the selectable traits associated with the agricultural-chemical-producing bacterium and the selectable traits associated with

the infecting bacterium may then be screened for their ability to interact with plant tissue in the manner in which the infecting bacterium interacts with plant tissue during the initial phase of infection of the plant host. As noted above, the initial phase of infection associated with Agrobacterium tumefaciens is binding to plant tissue cells. The ability of the hybrids to bind to plant tissue cells may be determined in vitro by techniques hitherto described in the above-cited literature and by techniques described in the following examples.

Other injecting bacteria, such as Erwinia stewartii, initially interact with plant tissue following infection by spreading throughout the vascular system of the plant without being detected and destroyed by the plant's disease-response system. This character is assumed in the literature to be due to an extracellular polysaccharide produced by the infecting bacterium which permits it to elude the plant's normal defensive reaction. The ability of the fusion hybrids to spread throughout the vascular system of the plant in this manner may be determined in any convenient manner. One preferred screening technique involves the infection of seedlings of the host plant at a particular site in the plant. After a period of time, e.g., four days, the plant may then be dissected into a plurality of transverse sections displaced along the longitudinal axis of the plant. Bacterial cultures may be regenerated from the bacteria contained in each section. The bacteria-containing sections farthest removed from the situs of initial infection will contain those fusion hybrids best able to disperse throughout the vascular system of the plant, thereby allowing selection of hybrids having this ability.

It is to be understood that other techniques for screening for this initial interaction with plant tissue may be envisioned by those skilled in the art in light of the teachings contained herein. It is to be further understood that other mechanisms of initial interaction between particular infecting bacteria and particular plant hosts may

be quantified and screened for in ways which will be obvious to those skilled in the art in light of the teachings contained herein and known interactions between infecting bacteria and their plant hosts.

The ability to spread throughout the plant's vascular system is a desirable property even for fusion hybrids formed from infecting bacteria whose initial interaction with plant cell tissue is by some other mechanism, e.g., cell binding. Accordingly, it is preferred to screen further those fusion hybrids found to possess both the ability to produce agricultural chemical and the ability to interact with plant cell tissue in the manner in which the infecting bacterium interacts with the plant host during the initial phases of infection to select a subgroup of hybrids for further screening according to the present invention which are also capable of dispersing quickly throughout the vascular system of the plant.

Moreover, in practicing this embodiment of the present invention, it is preferred that the fusion hybrids found to have both the ability to produce agricultural chemicals and the ability to interact with plant cell tissue in the manner in which the infecting bacterium interacts with plant cell tissue during the initial phases of infection be screened for these capabilities two or more times. Such repeated screening tends to assure stability of the fusion hybrid strain and also allows selection for further screening of a manageable number of fusion hybrids having the greatest capabilities in these two areas.

The stable fusion hybrids manifesting both the selectable traits of agricultural-chemical-producing bacterium and plant-tissue-interaction capabilities, as above described, may then be grown as individual colonies, in the case of the agricultural-chemical-producing bacterium that fix nitrogen, preferably on nitrogen-free media. Each of the resultant cultures may then be applied in an appropriate manner, e.g., by injection, to seedlings of the plant host in question. After an appropriate incubation period, e.g., two

or three weeks, those fusion hybrids which do not create any visible manifestation of a disease in the host plant seedlings may be selected for further screening. Experience has shown that screening of 25 to 30 of the best fusion hybrids is normally sufficient to provide 5 to 7 fusion hybrids which do not manifest visible disease symptoms, such as any associated with the original infecting bacterium, in the host plant in question.

Preferably, those stable fusion hybrids which manifest the widest area of spread throughout the vascular system of the plant, the clearest freedom from symptoms of disease in the host plant, and, in the case of agricultural-chemical-producing bacteria that fix nitrogen, the most vigorous growth in nitrogen-free media, are selected for further screening.

Some of the fusion products of the present invention have also been observed to form spores. Selection of endosymbiotic bacteria formed in accordance with the present invention which form spores facilitates application and survival of the bacteria when applied to plant hosts since the spores are generally more resistant to stress than the growing bacterial cells.

In another preferred embodiment, the agricultural-chemical-producing microorganisms of the present invention capable of entering into endosymbiotic relationships with a plant host are formed by the steps comprising:

(A) in any convenient order:
  (1) identifying the plant host; and
  (2) identifying an infecting microorganism which infects the plant host;
(B) preparing a vector capable of being transferred into and replicating in the infecting microorganism;
(C) preparing an expression module capable of directing the production of an agricultural chemical by the infecting microorganism;

(D) placing the expression module in the vector to create an expression vector capable of being transferred into and replicating in the infecting microorganism, the expression vector being capable of directing the production of the agricultural chemical in the infecting microorganism;

(E) transforming the infecting microorganism with the expression vector to produce hybrid microorganisms;

(F) selecting for the hybrid microorganisms;

(G) selecting serially from the selected hybrid microorganisms and in any convenient order:

(1) a subgroup comprising those hybrid microorganisms which manifest the ability to interact with plant tissue in the manner in which the infecting microorganism interacts with plant tissue during the initial phase of infection in the plant host;

(2) a subgroup comprising those hybrid microorganisms which, upon application to the host, do not create manifestations of disease; and

(3) a subgroup comprising those hybrid microorganisms having the ability to produce the agricultural chemical if not previously selected for; and

(H) selecting hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host by selecting from the products of the last performed step of steps (G)(1) to (G)(3) those hybrid microorganisms capable of improving the performance of the plant host under conditions wherein the performance would be improved by direct application of the agricultural chemical

or the agricultural-chemical-producing microorganism to the plant host.

As used in the steps above, the term "selecting" means any intervention or combination of interventions such that the desired microorganism can be recognized either by its ability to survive or by its unique properties.

A vector capable of being transferred into and replicating in an infecting microorganism may be prepared by one skilled in the art in view of the teachings of the present invention. As used herein, the term "prepared" includes obtaining existing vectors known to have the desired properties.

An expression module is prepared using techniques known in the art. As used herein, an "expression module" is a DNA sequence capable of directing the production of a product by a cell, in this case an agricultural chemical by the infecting microorganism. It comprises a portable DNA sequence containing the structural gene or genes for the production of an agricultural chemical and transcription and translation control elements, the control elements being operable in the infecting microorganism. The expression module may also contain a DNA sequence that codes for a selectable trait in the infecting microorganism. That sequence may be controlled by the transcription and translation control elements previously mentioned or it may have its own transcription and translation control elements that are operable in the infecting microorganism. It is preferred that the selectable trait be antibiotic resistance.

As used herein, the term "portable DNA sequence" is intended to refer either to a synthetically produced nucleotide sequence or to a restriction fragment of a naturally occurring DNA sequence. This sequence is obtained from the previously discussed agricultural-chemical-producing microorganisms. The methods of obtaining such sequences will be apparent to those of ordinary skill in the art in view of the teachings of the present invention and methods known in the art. Examples of such methods may be found in Maniatis,

T., et. al, Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory 198), which is specifically incorporated herein by reference in its entirety.

One preferred way of making an expression module is as follows. A portable DNA sequence containing the structural gene or genes for the agricultural chemical is prepared. The portable sequence is then cloned into a vector containing transcription and translation control elements for the portable DNA sequence. These control elements are operable in the infecting microorganism. If the portable DNA sequence and the control elements are properly aligned by techniques known to those skilled in the art, an expression module is created in this vector. The expression module is capable of directing the infecting microorganism to produce the agricultural chemical. The module is then recovered by techniques known in the art.

There are alternative ways of making an expression module. For example, some or all of the control elements may be attached to the portable DNA sequence prior to its being cloned into the vector, in which case the vector need not contain those elements.

The transcription and translation control elements, as discussed herein, include at least one promoter, at least one ribosome binding site, at least one translation initiation codon, and at least one translation termination codon. These elements may also include stability enhancing sequences and any other sequence necessary or preferred for appropriate transcription and subsequent translation of the vector DNA. These elements can include synthetic DNA, portions of natural DNA sequences, or products of in vitro mutagenesis. The particular control elements that are chosen for incorporation are chosen by criteria that are frequently empirically determined. That is, different configurations of control elements, for example, the ribosome binding site and its flanking sequences, are tested for their effect upon expression directly until a successful configuration is

evident. Such empirical determination involves techniques known to those skilled in the art.

After the expression module has been prepared, it is placed in the vector that is capable of being transferred into and replicating in the infecting microorganism, creating an expression vector. The expression vector is capable of being transferred into and replicating in the infecting microorganism and capable of directing the production of the agricultural chemical by that microorganism.

This vector is transferred into the infecting microorganisms to produce hybrid microorganisms by techniques known to those of ordinary skill in the art in view of the teachings of the present invention. Not all of the infecting microorganisms will be transformed by the vector. Therefore, it will be necessary to select for the hybrid microorganisms. Such selection techniques will be apparent to those of ordinary skill in the art in light of the teachings of the present invention. One of the preferred selection techniques involves incorporating into the expression vector a DNA sequence, with any necessary transcription and translation control elements, that codes for a selectable trait in the infecting microorganism, if such a sequence is not already in the expression module. A preferred selectable trait is antibiotic resistance.

Once the hybrid microorganisms have been selected, it is necessary to select for those that are hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host. This is done by following the steps of (G)(1)-(G)(3) and (H) above as described herein.

In a particularly preferred embodiment, the agricultural-chemical-producing DNA sequence is cloned into an integration vector, which is capable of integrating into the genome of the infecting microorganism. Use of such an integration vector prevents the transmission of the cloned DNA sequence to other microorganisms by plasmid transmission.

In particular, this method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host comprises:

(A) in any convenient order:
  (1) identifying a plant host; and
  (2) identifying an infecting microorganism which infects the plant host;
(B) preparing an integration vector containing an integration sequence and capable of integrating into the genome of the infecting microorganism;
(C) preparing an expression module capable of directing the production of an agricultural chemical by the infecting microorganism;
(D) placing the expression module within the integration sequence of the integration vector, thereby producing a modified integration vector capable of integrating into the genome of the infecting microorganism and directing the production of the agricultural chemical by the infecting microorganism;
(E) transforming the infecting microorganism with the modified integration vector to produce hybrid microorganisms;
(F) selecting for the hybrid microorganisms;
(G) selecting serially from the selected hybrid microorganisms and in any convenient order:
  (1) a subgroup comprising those hybrid microorganisms which manifest the ability to interact with plant tissue in the manner in which the infecting microorganism interacts with plant tissue during the initial phase of infection in the plant host;
  (2) a subgroup comprising those hybrid microorganisms which, upon application to

the plant host, do not create manifestations of disease; and

(3) a subgroup comprising those hybrid microorganisms having the ability to produce the agricultural chemical, if not previously selected for; and

(H) selecting hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host by selecting from the products of the last performed step of steps (G)(1) to (G)(3) those hybrid microorganisms capable of improving the performance of the plant host under conditions wherein the performance would be improved by direct application of the agricultural chemical or the agricultural-chemical-producing microorganism to the plant host.

As used in the steps above, the term "selecting" means any intervention or combination of interventions such that the desired microorganism can be recognized either by its ability to survive or by its unique properties.

The integration vector can be prepared by techniques known in the art. It must have a DNA sequence homologous to a natural DNA sequence from the organism whose genome is the integration target. This sequence is known as the integration sequence. In a particularly preferred embodiment, the integration vector is pCG300, which has been deposited in the American Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A. and assigned Accession No. 53329.

The expression module may be prepared as previously described herein or by other techniques known in the art. It should be noted that a selectable trait need not be in the expression module. It may be elsewhere in the integration vector, but it is preferably within the integration sequence.

For certain embodiments of the present invention, it is preferred that the expression module be prepared by:

1) preparing a vector containing a promoter that is operable in the infecting microorganism;
2) placing into the vector a portable DNA sequence containing the structural gene or genes for the production of the agricultural chemical and containing transcription and translation control elements other than the promoter, which are operable in the infecting microorganism, to produce an expression vector containing an expression module, wherein the expression module is operable in the infecting microorganism; and
3) recovering the expression module from the expression vector.

As previously mentioned, the control elements and the portable DNA sequence will need to be properly positioned by techniques known in the art in order to create an operable expression module.

The vector prepared by the first step in the preceding paragraph is often referred to as a promoter vector. Such a promoter vector is useful for moving a promoter from the infecting microorganism for the creation of the expression module. A particularly preferred promoter vector is the plasmid pCG6, which has been deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A. and given the Accession No. 53328.

As previously mentioned, there are alternative ways to make expression modules useful in the practice of the present invention that will be apparent to those skilled in the art in view of the teachings herein. In addition, the transcription and translation control elements may be obtained in several ways. They may be associated with the structural gene or genes in the portable DNA sequence, or they may also be created synthetically or by site-specific mutagenesis.

When the desired expression module is produced, it is removed and cloned into the integration vector, preferably within the integration sequence. The objective is that through double cross-over events flanking the expression module, only that portion of the integration vector comprising the expression module will be integrated into the genome of the infecting microorganism. In the event that the expression module has been cloned outside of the integration sequence, the entire integration vector may be integrated. In either event, the modified integration vector is capable of directing the production of the agricultural chemical by the infecting microorganism.

The modified integration vector is then used to transform the infecting microorganism to produce hybrid microorganisms. Since only some of the infecting microorganisms will be transformed, it is necessary to select for such hybrid microorganisms. There are many such selection techniques known to those of ordinary skill in the art. A preferred selection technique is to select for a marker or a trait such as antibiotic resistance in the hybrid microorganism.

Once they have been selected, the subgroups of hybrid microorganisms specified in steps (G)(1)-(G)(3) are selected for serially as described herein. Then, hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships for the plant host are selected by selecting those hybrid microorganisms capable of improving the performance of the plant host under conditions wherein the performance would be improved by direct application of agricultural chemical or the agricultural-chemical-producing microorganism to the plant as described herein.

For the embodiments of the present invention involving the use of recombinant DNA techniques, the plant hosts with which the hybrid microorganisms may establish endosymbiotic relationships, the infecting microorganisms, and the microorganisms that are the source of the agricultural chemical producing gene or gene include all of those

mentioned previously herein. However, it is preferred that the infecting microorganism and the microbial source of the agricultural chemical producing gene or genes are bacteria. Most preferably, the infecting bacterium is a species of the genus Corynebacterium or of the genus Clavibacter. Clavibacter xyli subsp. cynodontis and Clavibacter xyli subsp. xyli are especially preferred. When the infecting bacterium is a species of the genus Clavibacter, it is preferred that the plant host be of the Gramineae family. In this case, particularly preferred hosts include Bermuda grass, sugar cane, sorghum, and corn.

A preferred agricultural chemical is the delta endotoxin of Bacillus thuringiensis. Various Bacillus thuringiensis delta-endotoxin genes have been described, for example, in Klier, A., et al., The EMBO Journal, 7:791-799 (1982) and Schnepf, H.E., et al., Proc. Natl. Acad. Sci. USA, 5: 2893-2897 (1981), both of which are specifically incorporated herein by reference in their entirety. A preferred source of the gene for the delta toxin is the M13 vector mBTK65, deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under Accession No. 53326. It contains a truncated B. thuringiensis delta endotoxin gene fused to a kanamycin resistance gene in such a manner that both insecticidal activity and kanamycin resistance are expressed.

A preferred embodiment is a recombinant construction of Clavibacter xyli subsp. cynodontis ("Cxc") with Bacillus thuringiensis subsp. kurstaki ("Cxc/Bt"). In a particularly preferred embodiment, the Cxc harbors a chromosomally integrated plasmid which contains the gene encoding Bt. In another preferred embodiment, the plasmid encoding the Bt gene "reverts" or segregates itself from the chromosome of Cxc.

In another preferred embodiment of a Cxc/Bt recombinant construct, the Cxc does not have the capacity to transfer the introduced genetic material to other microbes.

In still another preferred embodiment, the Cxc/Bt recombinant declines rapidly in field soil. In a particularly preferred embodiment, the Cxc/Bt recombinant declines at the same rate of the wildtype microbe.

If not conducted in the initial screening of hybrid microorganisms, the hybrid organisms must, sooner or later, be screened directly for the ability to produce the agricultural chemical of interest. Preferably, the screening should be conducted at the earliest practical point in the process, which will be dictated by the number of hybrid microorganisms surviving at any particular stage of the process and the power of the technique used to screen for production of the agricultural chemical of interest to identify agricultural-chemical-producing hybrids out of a mixed inoculum.

It is to be understood that the screening for a particular agricultural-chemical-producing trait for use in accordance with the present invention will be within the capabilities of one having ordinary skill in the art through the exercise of routine experimentation in light of the teachings contained herein and inferences logically to be drawn from the disclosure of the present invention. The techniques contemplated as useful in screening for ability to produce the agricultural chemical may be determined by reference to standard compendia.

In general, these screening procedures will fall into one of four categories. These are (1) survival screens, where the agricultural chemical being produced is essential to survival of the hybrid and is not provided by the growth media; (2) analytical chemistry screens for the presence of the agricultural chemical being produced; (3) biological screens for the manifestations of biological activity associated with the agricultural chemical or chemicals being produced; and (4) immunoassay screens whereby organisms or groups of organisms producing a particular agricultural chemical are identified by interaction with an antibody which is specific to the chemical in question. The suitability of

any particular screening technique or combination of techniques will be dictated by the agricultural chemical in question. Thus, survival screens are particularly useful in identifying hybrid microorganisms capable of producing their own fixed nitrogen by survival on nitrogen-deficient media. Biological screens are particularly useful in identifying hybrid microorganisms capable of producing antibacterial or antifungal agents. A particularly preferred screening technique of this sort involves layering a culture of organisms susceptible to the antibiotic produced by the hybrid microorganism over a mixed culture of hybrids and identifying and removing for further screening hybrids or groups of hybrid producing that antibiotic from those areas where the susceptible organism cannot grow. Immunoassay techniques are particularly appropriate where the agricultural chemical of interest is a large molecule, such as a polypeptide. In any event, biological screens for the known effects of the agricultural chemical in question can be conducted using discrete cultures of hybrid microorganisms once the number of such cultures has been reduced to a manageable level, e.g., 10^2 - 10^3 cultures, by preliminary screens for other selectable traits associated with the agricultural-chemical-producing microorganism. Thus, hybrids producing insecticidal compounds, for example, could be identified by the inability of susceptible insects to survive on a culture of the hybrid. Such screening has the disadvantage of requiring screening of a larger number of colonies and requiring a protracted period of time to manifest results but is nonetheless capable of identifying hybrids with the ability to produce the agricultural chemical of interest.

It is to be understood that, with appropriate changes in the screening procedure, hybrids capable of fixing nitrogen anaerobically, as is done in Rhizobium, or in the presence of very small amounts of oxygen, as is done by microaerophilic bacteria, can be prepared in accordance with the present invention. Specifically, oxygen must be minimized or removed

in the nitrogen free growth medium during screening for nitrogen-fixing ability.

If such anaerobic nitrogen-fixing hybrids are to enter into endosymbiotic relationships with plant host, the hybrid must also contain genetic material, such as that contained in species of Rhizobium, which will cause the plant host to create an oxygen free or low oxygen environment not normally found in crop plants. For this reason utilization of aerobic nitrogen-fixing bacteria is preferred.

In order to determine which of the final group of hybrid microorganisms is capable of entering into endosymbiotic relationship with the host plant whereby agricultural chemicals produced by the hybrid may be utilized by the plant as a supplement to agricultural chemicals otherwise available, it is normally necessary to conduct preliminary yield gain screening or the like. For nitrogen-fixing hybrid bacteria produced by cell fusion, for example, this is accomplished by growing the host plant in an environment deficient in the agricultural chemical, e.g., fixed nitrogen, and comparing the dry weight after an appropriate period of growth, e.g., six weeks, with the dry weight of similar plants in similar environment but which had been infected, as by injection, with the stable fusion hybrid bacteria selected by the foregoing method. In the case of agricultural-chemical-producing bacteria that fix nitrogen, experience has shown that testing of 5 to 7 of the best fusion hybrids will result in identification of 3 or 4 stable fusion hybrids capable of creating statistically significant yield gains in the host plant growing in nitrogen deficient soil due, it is believed, to the ability of the stable fusion hybrids to fix atmospheric nitrogen aerobically and to enter into endosymbiotic relationships with the host plant.

It is envisioned that the endosymbiotic agricultural-chemical-producing microorganisms formed in accordance with the present invention may be further modified by natural or artificial genetic techniques to improve their performance as sources of agricultural chemicals for the plant host.

Specifically, the microorganisms may be modified to reduce their resistance to cold temperatures, thereby preventing unintended proliferation of the hybrid microorganisms from year to year. Similarly, the endosymbiotic hybrid microorganisms formed in accordance with the present invention may be modified to enhance their stability to drought, disease or other physiological stress. In this regard, the stability of the hybrid under stress conditions, and the ability to minimize the likelihood of spontaneous or forced reversion to a pathogenic state, is desired in a commercial microbiological product. In addition, the hybrid microorganisms could be modified to excrete the agricultural chemical. Ideally, the microorganism formed in accordance with the present invention should be modified so that they cannot grow outside the plant host, as by the selection of mutants requiring nutritional supplementation specifically or nearly specifically provided by the plant host in question. Techniques for genetic manipulation and mutant selection for strains of, for example, Azotobacter are well known in the art and are envisioned as being suitable for effecting the above-described and similar genetic manipulations of the stable hybrids formed in accordance with the present invention.

The stable hybrid microorganisms having the ability to produce agricultural chemicals and of entering into an endosymbiotic relationship with a plant host formed in accordance with the present invention may be used to prepare agricultural products in many ways. For example, hybrids formed in accordance with the present invention may be injected into seedlings or used to form coated seed of the plant host involved by association of the hybrid with a biodegradable nutrient carrier material which is coated on the seed. Similarly, hybrids formed in accordance with the present invention may be used to form infected seed products by application of the microorganisms directly to the seed of host plants. Alternatively, an agricultural soil drench may be prepared by mixing microorganisms formed in accordance

with the present invention with water and other appropriate drench constituents for application to the leaves, stem and roots of growing host plants and surrounding soil by spraying or the like.

It is to be understood that application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of processes within the scope of the present invention and the microbial products resulting therefrom appear in the following examples.

Example 1

Preparation of a Fusion Hybrid Bacterium From Azotobacter vinelandii and Erwinia stewartii Capable of Fixing Atmospheric Nitrogen Aerobically and of Entering Into an Endosymbiotic Relationship With Corn

Approximately 10^8 bacteria of the species Azotobacter vinelandii known to be capable of fixing nitrogen aerobically were selected. Approximately 10^8 bacteria of a strain of Erwinia stewartii mutants known to be resistant to both streptomycin and tetracycline were also selected. Fusion hybrids of these two types of bacteria were formed by a variant of the procedure of Weiss referred to above.

The bacteria growing in mid-log phase were treated with lysozyme at a concentration of 100 micrograms per milliliter and with DNA-ase at a concentration of 5 micrograms per milliliter in media containing EDTA at a concentration of 1 millimolar and magnesium chloride at a concentration of 5 millimolar, having a pH of 7 and with 12% added sucrose. After 10 minutes, protoplasts and spheroplasts of the original bacteria were formed. The temperature was then lowered to 0°C and the protoplast-forming solution was washed out. The resulting protoplasts were placed in 12% sucrose solution at 0°C. The solution was then supplemented with 40% polyethylene glycol of molecular weight about 6,000 in order to induce protoplast and spheroplast fusion for a period of

10 to 15 minutes. The fused bacteria were spun down in a centrifuge and resuspended in 12% sucrose plus a complete growth medium for 4 hours at 0°C. The temperature was then raised to 30°C and was held there for 2 hours, after which the hybrid fusion products were transferred to complete growth medium containing no additional sucrose for 2 hours.

The initial selection procedure was then conducted at a temperature of about 25-30°C.

The hybrid fusion products were transferred and grown on Burke's nitrogen-free medium as described in Carlson, et al., "Forced Association Between Higher Plant and Bacterial Cells in Vitro," Nature, Vol. 22, No. 5482, p. 393-395 (1974). The medium was nitrogen-free and contained added streptomycin at a concentration of 100 micrograms per milliliter and tetracycline at a concentration of 50 micrograms per milliliter. Only those fusion products manifesting both the ability to fix atmospheric nitrogen aerobically and manifesting the streptomycin and tetracycline resistance associated with the Erwinia stewartii strain originally introduced survived on this medium after a growth period of about 3 days.

Samples of the mixed fusion hybrids from this culture were used to infect corn seedlings approximately 3 to 4 feet in height by injection between the first and second node of the stalk tissue. After 4 days the corn plants were dissected into transverse sections to recover bacteria that moved to the anterior (top) of the plant, since Erwinia stewartii interacts with plant tissue during the initial phase of infection by spreading throughout the vascular system of the plant without being recognized or destroyed by the plant's disease-response system. Those fusion hybrids with the greatest facility to spread throughout the vascular system of the corn plant were generally found between the fifth and sixth node.

The fusion hybrid bacteria found between the fifth and sixth node of the corn plant were cultured for 3 days on Burke's nitrogen-free medium as described by Carlson, et al.,

supra. After 3 days, a mixed inoculant from this nitrogen-free culture was again used to infect corn seedlings approximately 3 to 4 feet in height by injection between the first and second nods of the stalk tissue. After about 3 days the plant was transversely dissected as above described to recover bacteria that moved to the anterior of the plant. Again, those fusion hybrids with the greatest facility to spread throughout the vascular system of the corn plant were generally found between the fifth and sixth node.

The fusion hybrid bacteria recovered from this portion of the corn plant were then grown as separate colonies on Burke's nitrogen-free medium. Twenty-five of the most vigorous colonies were selected, and each was used to infect one of 25 corn plants by injection. After 3 weeks, those colonies which did not result in any visible manifestation of disease in the infected corn plants were selected. From among these, seven colonies which manifested the most vigorous growth on nitrogen-free media, the widest area of spread throughout the plant and least manifestation of disease symptoms were selected for preliminary yield testing and were identified as Fusion #1 through Fusion #7.

In the preliminary yield test, flats (30cm x 46cm) containing 15cm of washed sterile sand over 10cm of Perlite were sown with 24 corn seeds at a uniform spacing. Flats were maintained in the greenhouse (at approximately 22-27°C) and were watered twice daily with deionized water. Starting at 10 days (so as to exhaust the seed nitrogen) the seedlings were watered three times a week with a nutrient solution. One liter of solution was applied and allowed to drain through. The solution was a modified Long Ashton mix that contained nitrate ion at a concentration of 6 micromolar as its sole nitrogen. The Long Ashton mix was also modified to contain nitrate ion at concentrations of 6 and 0 millimolar and used in comparative runs A and C below, respectively. Experiments were run for six weeks, at which time the aerial portions of the plants were harvested and dried for one week at 95°C. Dry weight data was then collected. For flats

containing infected plants, the bacterial strains were introduced by injection at 10 days (at the same time as fertilization was initiated). Each analysis was done in triplicate (three flats of 24 plants per treatment) and that data were subjected to statistical analysis. Seven different bacterial fusions were selected by multiple screens between Erwinia stewartii and Azotobacter vinelandii (Fusion #1 through #7) as above described and used in this work. Actual dry weight yield data per flat (average of the three flats) is as follows:

Inoculation	NO_3 Level	Average Flat Dry Weight (grams)	Percentage Increase Over Control
A. None	6 millimolar	116.8	
B. None (Control Value)	6 micromolar	34.8	
C. None	None	19.2	
Fusion #1	6 micromolar	44.8	29
Fusion #2	6 micromolar	39.6	14
Fusion #3	6 micromolar	72.4	108
Fusion #4	6 micromolar	33.2	-5
Fusion #5	6 micromolar	54.8	57
Fusion #6	6 micromolar	25.2	-28
Fusion #7	6 micromolar	56.8	63

Results with Fusion numbers 1, 3, 5, and 7 are statistically significant.

Uninoculated comparative run A contains the optimum level of nitrate in Long Ashton nutrient solution and is indicative of results to be expected with corn growing in well nitrogen-fertilized soil. Comparative run C, to which no nitrate was added, shows the yields which may be expected based only on residual nitrate contained in the seed of the corn. Comparative run B, which represents the control value containing nitrogen at levels of about 6 micromolar, is indicative of growth to be expected in poorly nitrogen-fertilized soil. The reduction in dry weight associated with fusion product number 6 is apparently due to non-visible

pathogenic effects, even though none of the plants manifested visual symptoms of disease. The results of fusion product number 4 are not statistically significant. The results of fusion products 1, 3, 5, and 7 show that the process of the present invention was able to produce at least four stable fusion hybrid products capable of fixing atmospheric nitrogen aerobically and capable of entering into endosymbiotic relationships with corn whereby corn growing under nitrogen stressed conditions in conjunction with the fusion products of the present invention produced yields of from 29% to 108% greater than a control without the bacteria formed in accordance with the present invention.

Example 2

Preparation of a Fusion Hybrid Bacterium from Azotobacter vinelandii and Agrobacterium tumefaciens Capable of Fixing Atmospheric Nitrogen Aerobically and of Entering Into an Endosymbiotic Relationship with Sugar Beets

Approximately 10^8 bacteria of the species Azotobacter vinelandii were selected. Approximately 10^8 bacteria of a strain of Agrobacterium tumefaciens mutants which are resistant to both streptomycin and kanamycin were selected. Protoplast fusion was effected between these bacteria as in Example 1. The fused protoplasts were transferred to Burke's nitrogen-free medium as described in Example 1 supplemented with streptomycin at a concentration of 100 micrograms per milliliter and kanamycin at a concentration of 50 micrograms per milliliter. After 3 days, only those stable fusion hybrids capable of both fixing atmospheric nitrogen aerobically and possessing the genetic material from the pathogenic bacteria associated with streptomycin and kanamycin resistance survived.

A mixed inoculum from this culture was introduced into a suspension culture of carrot tissue cells in the manner described by A.G. Matthysse, et al., Physiological Plant Pathology, 20, 27-33 and 21, 381-387 (1982), which is specifically incorporated herein by reference in its

entirety. After about 3 hours, the carrot cells and those fusion hybrids capable of binding to carrot cells in a manner similar to the initial interaction of Agrobacterium tumefaciens with plant tissue cells during the initial phases of infection were separated from unbound bacteria by washing 3 times in sterile, distilled water. The carrot cells with adhering hybrid bacteria were macerated and washed again. The resulting suspension was spun down in a centrifuge and the resulting pellet, consisting of cell walls and adhering hybrid bacteria, was placed on Burke's nitrogen-free medium for 3 days.

The cell-binding method as above-described was repeated and the fusion hybrids thus selected were grown as separate colonies on nitrogen-free media. After 3 days of growth, 25 of the most vigorous colonies were selected.

Each colony was used to infect one of 25 sugar beet seedlings 8 to 10 inches high by insertion of a pin, which had been dipped into the colony, into the area of the hypocotyle between the root and the plant. Those hybrid colonies which did not form a gall or tumor in the sugar beet seedling after 3 weeks were selected for further screening.

The roots of each infected sugar beet plant which did not manifest any visible symptoms of disease after 3 weeks were then dissected into a number of transverse sections. The distance down the root of the plant to which the hybrid bacteria had spread was noted. Five of the 25 stable fusion hybrids were selected for preliminary yield testing based upon the vigorousness of their growth on nitrogen-free media, the absence of visible manifestations of disease associated with their injection into the plant and the facility with which they were able to spread throughout the plant tissue. These five fusion hybrids, designated Fusion #1 through Fusion #5 were subjected to preliminary yield testing as follows:

Flats (30cm x 46cm) containing 15cm of washed sterile sand over 10cm of Perlite were plated with 24 germinated sugar beet seeds at a uniform spacing. Flats were maintained

in the greenhouse (at approximately 22-27°C) and were watered twice daily with deionized water. Starting at 10 days (so as to exhaust the seed nitrogen) the seedlings were watered three times a week with a nutrient solution. One liter of solution was applied and allowed to drain through. The solution was a modified Long Ashton mix that contained nitrate ion at a concentration of 6 micromolar as its sole nitrogen. The Long Ashton mix was also modified to contain nitrate ion at concentrations of 6 and 0 millimolar and used in comparative runs A and C below, respectively.

Experiments were run for six weeks, at which time the plants were harvested and dried for one week at 95°C. Dry weight data was then collected. For flats containing infected plants, the bacterial strains were introduced by injection at 10 days (at the same time as fertilization was initiated). Each analysis was done in triplicate (three flats of 24 plants per treatment) and the data were subjected to statistical analysis. Five different bacterial fusions were selected by multiple screens between Agrobacterium tumefaciens and Azotobacter vinelandii as above described and used in this work. Actual dry weight yield data per flat (average of the three flats) is as follows:

Inoculation	NO_3 Level	Average Flat Dry Weight (grams)	Percentage Increase Over Control
A. None	6 millimolar	44.7	-
B. None (control value)	6 micromolar	8.8	-
C. None	None	5.2	-
Fusion #1	6 micromolar	8.0	-9
Fusion #2	6 micromolar	12.4	41
Fusion #3	6 micromolar	6.9	-22
Fusion #4	6 micromolar	24.6	180
Fusion #5	6 micromolar	14.9	69

Results with Fusion numbers 2, 3, 4 and 5 are statistically significant.

Uninoculated comparative runs A, B, and C have the same significance as uninoculated comparative runs A, B, and C in Example 1 above. Fusion product No. 3 appeared to result in non-visible pathogenic effects, even though no visible symptoms of disease were observed on any of the plants. The preliminary yield data confirms that the process of the present invention was capable of producing at least three stable hybrid bacteria (Fusion #'s 2, 4 and 5) capable of fixing atmospheric nitrogen aerobically and of entering into an endosymbiotic relationship with sugar beets whereby sugar beets growing in nitrogen stressed conditions generated yields 41 to 180% in excess of those obtained with uninoculated sugar beets.

Example 3

Example 2 was repeated with the exception that the fusion products were applied to sugar beets receiving nitrogen in the form of nitrate at a concentration of 6 millimolar -- that associated with optimal nitrogen fertilization. No significant yield gain above that attained by uninoculated comparative run A was observed in the inoculated sugar beets. These results confirm that the yield gains associated with the fusion products in Example 2 were due to an endosymbiotic relationship between the fusion products and the plant whereby fixed nitrogen was provided by the fusion products and used by the plant host. Had the yield gains reported in Example 2 been the result of growth regulators or hormones provided by the fusion products, similar gains should have been observed in Example 3.

Further evidence of an endosymbiotic association with the host is provided by light microscopy using gram stain and from reisolation of the bacteria (with the expressions of the maintained antibiotic resistance markers) from infected plants. Moreover, the hybrid bacteria can be found throughout the plant. Their location is not limited to the site of infection. The number of hybrid bacteria in plant tissue was approximately 10^5 per gram (dry weight) in a corn plant growing under low nitrate conditions.

It is to be understood that, with appropriate changes in the screening procedure, hybrids capable of fixing nitrogen anaerobically, as is done in Rhizobium, or in the presence of very small amounts of oxygen, as is done by microaerophilic bacteria, can be prepared in accordance with the present invention. Specifically, oxygen must be minimized or removed in the nitrogen-free growth medium during screening for nitrogen-fixing ability.

If such anaerobic nitrogen-fixing hybrids are to enter into endosymbotic relationships with plant hosts, the hybrid must also contain genetic material, such as that contained in species of Rhizobium, which will cause the plant host to create an oxygen free or low oxygen environment not normally found in crop plants. For this reason utilization of aerobic nitrogen-fixing bacteria is preferred.

Some of the fusion products of the prevent invention have also been observed to form spores. Selection of endosymbiotic bacteria formed in accordance with the present invention which form spores facilitates application and survival of the bacteria when applied to plant hosts since the spores are generally more resistant to stress than the growing bacterial cells.

Example 4

Production of a Fusion Hybrid Expressing Invertebrate Pesticide Activity for Use in Sweet Potatoes

The streptomycete S. ipomoea is a pathogen of Ipomoea species, which includes morning glories and sweet potatoes (Ipomoea batata). This organism invades both the fleshy root and fibrous roots of the latter, and is an endophyte candidate for fusion with an appropriate agchemical producing bacterium. Such a partner is S. avermitilis, which produces a family of compounds called avermectins, which are effective against a variety of insect pathogens, such as nematodes, mites, and insects. Campbell, W. C. et al., Science 221: 823-828 (1983). Using the technique of protoplast fusion, hybrids of S. ipomoea and S. avermitilis were produced, which

can associate with sweet potato tissue and produce avermectins.

Spores (10^8) of S. avermitilis strain SA-5 (nic) on deposit with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Maryland 20852 USA under Accession No. 53319 and S. ipomoea wild type SI-1 on deposit with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Maryland 20852 USA under Accession No. 53320 were inoculated into, respectively, 30 ml of YSG 1 and YSG 2 broth (modified YSG 1 with 14% sucrose and 0.2% glycine). YSG 1 was prepared according to the method of Chater, K.F., et al., Cur. Top. Micro. Bio. Immunol. 96: 69-95 (1982), specifically incorporated herein by reference in its entirety. Both cultures were incubated at 30° with vigorous aeration (240 rpm) for 24 hours. Protoplasts were prepared from both strains and suspended in protoplast buffer med P as described in Hopwood, D.A., et al., Mol. Gen. Genet. 162: 307-317 (1978), specifically incorporated herein by reference in its entirety. SI-1 protoplasts were irradiated with UV light at 254 nm (Model UVGL-58 Mineralight Lamp, UVP Inc., San Gabriel, California) at 8 cm from the source for 2 minutes to give 1% cell survival. UV-irradiated SI-1 protoplasts (10^7) were mixed with 10^7 SA-5 protoplasts and sedimented by centrifugation at 6000 rpm (Sorval SS-34 rotor) for 5 min at 4°C. The pelleted protoplasts were suspended gently in 0.1 ml of med P, and 0.9 ml of 40% (wt/vol) PEG 8000 (Sigma) in med P was added followed by gentle mixing. After incubation at 25°C for 3 minutes, the PEG-treated protoplasts were diluted 10-fold with med P, centrifuged and suspended in 2 ml of med P as previously. Samples (0.1 ml) of the fused protoplasts were plated at 30°C onto protoplast regeneration plates (RM16: yeast extract 0.2%, casamino acids 0.01%, sucrose 15%, glucose 1%, oatmeal 0.3%, L-proline 0.1%, $MgCl_2.2H_2O$ 1%, Na_2SO_4 0.025%, agar 2%, trace element (Hopewood, D.A., op. cit.) 0.2%. After autoclaving the following sterile solutions were added: $CaCl_2$ 20 mM; KH_2PO_4 5 mM, MES (2-(N-Morpholino) ethane sulfonic acid) buffer (pH

6.5), 25 mM). The regenerated colonies were replica-plated by cellulose acetate membrane filters (pore size 0.45um, diameter 82 mm, Micro Filtration System, Japan) onto minimal agar HMM plates (Hopwood, D. A., Bacterial Rev., 31: 373-403 (1967)) to screen for prototrophs. The latter were subsequently patched onto YDC plates yeast extract 0.4%, malt extract 1%, dextrose 0.4%, maltose 0.4%, agar 1.5%. After autoclaving, $MgSO_4$ (10 mM) and CaCl 6.25 mM were added. The plates were incubated for 7 days until the cells had sporulated, and those with SI-1 colony morphology and inability to produce melanin on YDC plates were further screened for avermectin activity by a toxicity test using freshly hatched brine shrimp. A 1 mm^2 piece of colony was placed in the well of a 96-well microtiter dish (nunc) and 0.01 ml of methanol added. Brine shrimp (ca. 10 per 0.1 ml) were added and the time of paralysis noted, and compared to controls. Strain SI-1 gave no paralysis in this test compared to control; strain SA-5 produced complete paralysis in 15 minutes at 22°C.

Among 10^5 regenerated colonies, 270 showed SI colony morphology. Ten percent of the latter exhibited avermectin activity (paralysis within one hour), and 6 of these positive clones were further inoculated onto sweet potato slices to assay for their ability to associate and grow in plant tissue. Moyer, J.W. et al., Phyopathology, 74: 494-497 (1984). All 6 grew on sweet potato slices, and 2 of these recovered from the plant tissue retained avermectin activity.

Example 5

Endophyte Hybridization with Recombinant DNA

The bacterial species Clavibacter xvli subsp. cynodontis (Cxc) and subsp. xvli (Cxx) are vascular inhabitors of Bermuda grass and sugar cane, respectively, Davis, M.J. et al., Int. J. Syst. Bacteriol., 34: 107-117 (1984), which is specifically incorporated herein by reference in its entirety. They can grow in other commercially valuable crops such as sorghum. Liao, C.H., et al., Phytopathology, 71:1303-1306 (1981), which is specifically incorporated

herein by reference in its entirety. It has been discovered that Cxc and Cxx can also grow in field and sweet corn without pathogenic effect. The following example teaches how the organism Cxc can be modified to produce an insecticidal protein, the Delta endotoxin of Bacillus thuringienesis var. Kurstaki HD-73, which is active upon lepidopterous insects such as corn earworm (Heliothus zea) and European corn borer (Pyrausta nubilalia), which are pests of field and sweet corn.

Preparation of Integration Vector

The genes for agricultural chemicals are preferably carried on an integration vector for Cxc to prevent transmission of cloned genes to other organisms by plasmid transmission. Integration vectors have the foreign gene sequences inserted into or adjacent to a natural sequence of DNA from the organism whose genome is the integration target. See Saunders, C.W. et al., J. Bacteriol. 157: 718-726 (1984). The integration vector is commonly propagated in a permissive host such as E. coli or B. subtilis.

The manipulation of the foreign agricultural chemical gene sequences is carried out in E. coli or B. subtilis using replication vectors for these hosts. When the desired gene configuration is produced, the configuration is removed and cloned into the Cxc integration vector adjacent to or within the Cxc cloned DNA segment, and the integration vector is propagated in the permissive host. Purified integration vector DNA is then used to transform the desired Cxc host, selecting for a marker such as drug resistance, which is also carried in the foreign gene configuration. Since the vector does not carry a replication origin for propagation in Cxc, the vector must recombine with the Cxc chromosome in order to persist in Cxc. Recombination is facilitated by the Cxc homologous sequence on the integration vector. The stable drug-resistant transformants are then screened for the production of the agricultural chemical gene products, e.g. B. thuringenesis endotoxin.

Recombinant DNA Methods

The restriction enzymes, T4 DNA ligase and calf intestinal alkaline phosphatase, used in the following methods were purchased from New England BioLabs, Bethesda Research Laboratories, or Boehringer Mannheim. The reaction conditions were those recommended by manufacturer.

Isolation of TBIA DNA

A 3 litre culture of Clavibacter xyli subsp. cynodontis (TBIA, on deposit with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Maryland 20852 U.S.A. under Accession No. 33973) was grown aerobically in S8 medium (Davis, M. J., et al., Science 210: 1365-1367 (1980)) to mid-exponential stage at 28°C, at which point glucose and glycine were added to final concentrations of 2% and 0.1%, respectively. After growth for 17 hours, the cells were centrifuged, washed with lysis buffer (50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA) and resuspended in 2 ml of the same. The cells were then lysed by addition of guanidine hydrochloride and sarkosyl to final concentration of 7M and 4%, respectively, and Tris-Cl (pH 8.0) to 20 mM, and EDTA to 20 mM. Sung, M.T., et al., Genetic Engineering in the Plant Sciences, N.J. Parapoulos (ed.), pgs. 39-62 (Praeger Scientific Press 1981), specifically incorporated herein by reference in its entirety. The final volume was 20 ml. After overnight incubation at 50°C, 4 ml of distilled water was added to the lysed suspension and the mixture spun at 12,000 rpm for 10 minutes at 20°C in the SS34 Beckman rotor. This was done to separate cell wall and membrane debris from the nucleic acids remaining in the supernatent. The supernatent fluid was mixed with ethidium bromide (final concentration equal to 600 ug/ml) and applied to a 2-step cesium chloride gradient which was centrifuged for 24 hr., 26000 rpm at 20°C in a Beckman SW27 rotor. The top step consisted of 4.42 M CsCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA to a final density of 1.55; the bottom step consisted of 5.7M CsCl, 0.02M Tris-Cl (pH 8.0), to a final density of 1.7. The DNA band was extracted and purified by standard techniques.

Maniatis, T., et al., Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory 1982), specifically incorporated herein by reference in its entirety.

TBIA Chromosomal Fragment Isolation

TBIA chromosomal DNA was partially digested with the restriction enzyme, Sau3Al, resulting in DNA fragments ranging from approximately 1 kb to 25 kb (as shown by 0.7% agarose gel electrophoresis). DNA fragments approximating 10 kb were isolated by centrifuging the partial Sau3Al digest of TBIA DNA through a sucrose gradient. M. R. El-Gewely and R. B. Helling, Anal. Biochem. 102:423-428 (1980).

Cloning of TBIA DNA Fragment

The cloning vector, pUC19 (New England Biolabs 1985-86 Cata-logue, p.90-91) was digested with the restriction enzyme, BamHI, to create a linear plasmid with cohesive ends. To prevent self-religation, the ends were treated with calf intestinal alkaline phosphatase. Maniatis, T., et al., op. cit. The Sau3Al digested TBIA fragments approximating 10 kb (isolated by above procedure) were then ligated into the unique BamHl site of pUC19 using T4-DNA ligase. The concentrations of vector (pUC19) and insert (Sau3Al digested TBIA fragments) used to obtain the correct circular constructions were 0.0044 and 0.0156 ug per microliter of ligation mix, respectively. Dugaiczyk, A., et al., J. Mol. Biol. 96: 171 (1975); Helfman, D.M., et al., Focus vol. 6, No. 1 (Bethesda Research Laboratories 1984). The ligation was carried out with T4 DNA ligase at 0.05 units per microliter of ligation mix at 14°C for 48 hours. The ligation mixture was then transformed into competent E. coli JM109 cells (on deposit with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Maryland 20852 USA under Accession No. 53323), using the calcium chloride procedure. Maniatis, T., et al., op. cit. Transformants with inserts were identified as ampicillin resistant white

colonies in the presence of Xgal. Vieira, J. and Messing, J., Gene 19: 259 (1982).

Screening of Transformants

Positive transformants (ampicillin resistant white colonies) were screened for large TBIA DNA inserts (equal to or greater than 10kb) which contained unique restriction sites near or at the middle of the insert. Transformants were lysed by the alkaline lysis plasmid DNA mini-prep method (Maniatis, T., et al., op. cit.) and subjected to restriction enzyme digests. A preferred plasmid from this screening was pCG300 (on deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under Accession No. 53329), which contains a 13 kb TBIA DNA insert with one EcoR1 site near the middle of the insert (Fig. 1).

Cloning of Kanamycin Resistance Gene

In order to determine that the EcoRI site in the TBIA insert of pCG300 is not located in a critical TBlA gene, a kanamycin resistance cassette (Pharmacia 1984 Catalogue Molecular Biologicals, Chemicals and Equipment for Molecular Biology p. 74) was cloned into the site and this DNA transformed into TBIA, selecting for kanamycin resistant integrates. The recombinant plasmid, pCG300, was partially digested with the restriction enzyme EcoRI, and ligated (using T4 DNA ligase), with the EcoRI DNA fragment containing the kanamycin resistance gene from Tn903. Dugaiczyk, A., et al., J. Mol. Biol. 96:171 (1975); Pharmacia 1984 Catalogue Molecular Biologicals, Chemicals and Equipment for Molecular Biology p. 74. The ligation mixture was incubated at 14°C for 72 hours. The ligation mixture was then transformed into competent E.coli strain SK2267 (on deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 USA under Accession No. 53324); transformants were selected on plates containing kanamycin sulfate (50 ug/ml). Kanamycin resistant transformants were screened for the favored construct -- the kanamycin gene cloned into the EcoRI site found in the TBIA DNA insert of pCG300. The new plasmid, pCG306 (Fig. 2), is then used to transform TBIA

cells, selecting for kanamycin resistance. The finding of stable transformants shows that the EcoR1 site can be used for integration of agricultural chemical genes without compromising the growth of TBlA.

Cloning of promoters from TBIA

In order to insure that foreign genes will be expressed in TBIA, it is preferable to clone regulatory sequences from TBIA for fusion to foreign gene sequences.

Sau3Al fragments from a partial restriction digest of TBIA chromosomal DNA (see above) were cloned (Maniatis, T., et al., op. cit.) into the BamHI site of a Bacillus promoter cloning plasmid, pPL703 (on deposit with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Maryland 20852 U.S.A. under Accession No. 53327) which was derived from pPL603. Williams D. et al., J. Bacteriol. 146: 1162-1165 (1981). This plasmid (Fig. 3) contains a structural gene for chloramphenicol acetyltransferase (CAT) which is not expressed due to lack of a promoter. Recombinant plasmids were transformed into competent Bacillus subtilis 62037 (rec E) (on deposit with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Maryland 20852 U.S.A. under Accession No. 53325). Restriction fragments that promote expression of chloramphenicol acetyltransferase gene were identified by selecting chloramphenicol resistant transformants. Twenty-one chloramphenicol resistant colonies were obtained, and their chloramphenicol acetyltransferase activities were measured. Shaw W. V., in Methods in Enzymol. 43: 737-755 (Academic Press 1975). The transformants contained recombinant plasmids with TBIA DNA insertions ranging from 0.4 to 2 kilobases and chloramphenicol acetyltranferase activity ranging from 49 to 1026 nmole/min/mg protein. The plasmid pCG6 (Fig. 3) (on deposit with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Maryland 20852 U.S.A. under Accession No. 53328) with the highest promoter activity for chloramphenicol acetyltransferase is the preferred source of regulatory signals for the expression in TBIA of desired agricultural

chemical proteins, e.g. Bacillus thuringiensis insecticidal endotoxin.

Expression of B. thuringiensis Delta-Endotoxin from a Cxc Promoter

The M13 vector mBTK65 (on deposit with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Maryland 20852 U.S.A. under Accession No. 53326) contains a truncated B. thuringiensis delta-endotoxin gene fused to a kanamycin resistance gene in such a manner that both insecticidal activity and kanamycin resistance are expressed. The fusion sequence is bounded by a BglII site 5-prime to the Shine-Delgarno (ribosome binding site) sequence and a HindIII site 3-prime to the kanamycin resistance gene (Fig. 4). This fusion hybrid sequence can be ligated 3-prime to a Cxc promoter (as in pCG6) to bring expression of both activities under Cxc control. The procedure is as follows: Phage mBTK65 replicative form (RF) DNA is prepared from ATCC No. 53326 by standard methods. Barnes, W.M., et al., in Methods in Enzymology, 101: 98-122 (Academic Press 1983). The purified DNA is cut sequentially with the restriction enzymes Bg1II and HindIII to liberate a 2.64 Kb fragment, which is isolated and purified by preparative agarose electrophoresis as described in Maniatis, T., et al., op. cit. The purified fragment is then blunted by treating with the klenow fragment of DNA polymerase as described in Maniatis, T., et al., op. cit.

In order to optimize the translation of the delta endotoxin-kanamycin resistance fusion gene, the promoter segment of pCGC6 is treated with the exonuclease Bal-31 to generate multiple 3-prime termini for fusion to the Shine-Dalgarno sequence of the gene fusion. Roberts, T.M., et al., Proc. Natl. Acad. Sci. USA, 76: 760-764 (1979); Deans, R., et al., in Recombinant DNA Techniques, 2:2-6 (The University of Michigan 1981). In order to select kanamycin resistance generated by the fusion hybrid, it is necessary to inactivate the neomycin (kanamycin) resistance element already present in pCG6. This is done by cutting pCG6 with BglII

endonuclease, which cute within the neomycin resistance gene; the BglII ends are then filled in with the Klenow fragment of DNA polymerase and the blunted vector is recircularized with T4 ligase. Maniatis, T., et al., op. cit. The ligated DNA is transformed into B. subtilis strain 62037 as before, selecting chloramphenicol resistance and screening for kanamycin sensitivity, indicating that a frameshift has resulted in inactivation of the neomycin resistance element. This plasmid is designated pCG6 Neo^S. Following digestion of pCG6 Neo^S with SalI, the DNA is subjected to Bal-31 as described (Deans, R., op. cit.) (Fig. 5) to generate a family of deletions, whose pattern is monitored by gel electrophoresis. The heterogeneous plasmid fragment population is then ligated with the blunted BglII-HindIII fragment, above. The ligated DNA is transformed into B. subtilis 62037 (rec E), selecting for kanamycin resistance. Since the expression of kanamycin resistance in the gene fusion is dependent on the transcription and translation of the 5-prime delta-endotoxin gene, only favorable configurations of promoter and the delta-endotoxin gene fusion will give rise to kanamycin resistance. The kanamycin resistance transformants are screened by standard recombinant DNA techniques (Maniatis, T., et al., op cit.) to verify the restriction map. The expression of delta-endotoxin activity is monitored by subjecting freshly-hatched larvae of the tobacco hornworm (Carolina Biologicals) to samples of the colony incorporated into their standard diet. Kanamycin resistant transformants found to produce effective levels of larvae toxicity are grown up and their plasmid DNA extracted. The plasmid DNA is cut by SmaI and HindIII restriction endonucleases which cut out the promoter-gene fusion region (cf. Fig 5) and the ends blunted as before. This fragment is then cloned into the integration vector pCG300 at the blunted EcoRI site by standard methods (Maniatis, T., et al., op. cit.). The ligated DNA is transformed as previously into E. coli SK2267, selecting for kanamycin resistance. Transformants from the latter procedure are screened as

before for insecticidal activity and expected restriction map. Transformants giving the correct restriction map and insecticidal activities are then grown up for plasmid DNA preparation and subsequent transformation of TBIA.

Preparation of TBIA protoplasts

TBIA protoplasts are prepared by the following method:

1) inoculate TBIA from frozen glycerol stock into S8 medium;
2) aerate the culture at 30°C until mid-exponential;
3) add 2% glucose, 0.1% glycine to the culture, and continue aeration overnight (approximately 17 hours);
4) spin down 20 ml of culture in sterile tubes at 8000 rpm for 5 minutes at 4°C, wash once in 10 ml of SMMC buffer (sorbitol 0.5M - 20 mM maleate - 20 mM $MgCl_2$ - 20 mM $CaCl_2$ (pH 7.0) (Yoshihama M., et al., J. Bacteriol. 162: 591-597 (1985)) and suspend in 2 ml of SMMC buffer containing 2.5 mg/ml lysozyme;
5) incubate at 30°C for 2-3 hours, and check for the formation of protoplasts under a microscope; and
6) harvest cells by centrifugation at 6000 rpm for 10 minutes at 4°C, wash once with 5 ml of SMMC buffer, and suspend in 1 ml of SMMC buffer.

Transformation of TBIA protoplasts

A 20 ml exponential culture of TBIA growing in S8 medium is pretreated with 2% glucose and 0.1% glycine overnight (approximately 17 hours), and protoplasts are prepared as described above. Samples (0.3 ml) of protoplasted cells are placed in tubes, and the integrative plasmid DNA carrying the TBIA promoter and B. thuringiensis delta endotoxin fusion gene (1 ug DNA in 10 ul of 0.5 M sorbitol (pH 7.0)) is added. Then 0.7 ml of 25% PEG in SMMC buffer is added, followed by gentle mixing. After incubation at 25°C for 5 min., the mixture is diluted 10 fold with SMMC, centrifuged as above, suspended in 1 ml of SSC broth (S8 broth with 0.5 M sorbitol (pH 7.0)), and incubated with shaking at 30°C for 2 hrs.

The transformed protoplasts (0.1 ml) are plated onto cellulose acetate membrane filters on top of nonselective regeneration plates (SSC), and incubated for 2-3 days at 30°C to allow expression of kanamycin resistance. The filters are then transferred to selective regeneration plates (SSC + kanamycin (50 ug/ml)), and incubated further at 30°C to select for kanamycin resistant transformants.

Test for Larvacidal Activity of Hybrid TBIA in Corn

Kanamycin resistant transformants (above) are then grown in S8 medium plus 50 ug/ml kanamycin and portions of the culture tested for larvacidal activity in vivo as described above. The larvacidal isolates are then inoculated into corn seedlings (FR 632, Illinois Foundation Seeds). Inoculations are performed by piercing 2 - 3 week old seedlings with a pointed sterile scapel which has been dipped in a colony to be tested. Periodically during the life cycle of the plant, xylem sap samples are removed and tested for kanamycin resistant hybrids which are larvacidal in vivo. As stated elsewhere in this document, isolates that routinely show colonization of the corn without disease symptoms, and which express the agricultural chemical B. thuringiensis endotoxin) are selected for tests of in vivo efficacy. The latter test is performed by challenging inoculated corn plants with freshly hatched larvae of the corn earworm or the European corn borer.

By way of further example of agricultural-chemical-producing microorganisms that can be used as sources of genetic material for use in the foregoing procedure, such microorganisms may contain genes encoding antibiotics synthesized by plants, invertebrates and vertebrates. These agricultural-chemical-producing microorganisms include those in which the agricultural chemical gene has been introduced by genetic engineering means. Non-limiting examples of these agricultural chemicals include lysozymes, 1,3-glucanases, chitinases, cecropins, attacins, and magainins. Also included are animal and plant derived protease inhibitors. These inhibitors are known to increase plant defense

responses, or to enhance the insecticidal activity of proteins such as those produced by Bacillus thuringiensis. Trypsin inhibitors from the cowpea plant (Vigna unguiculata) and other legumes are effective antimetabolites for control of insects. A polypeptide isolated from tomato leaves, and referred to as systemin, induces the synthesis of wound-inducible protease inhibitors in tomato plants.

The genes encoding the agricultural chemicals discussed above can be used according to the methods of the present invention to transform an infecting microorganism. However, these methods can be employed to transform an infecting microorganism with a gene encoding any proteinaceous crop protection agent produced by microorganisms, plants, vertebrates, or invertebrates.

In this example, methods are described for expressing genes encoding eukaryotic proteins as crop protection agents in recombinant bacteria. The general methods for preparing an integration vector and transfecting a microorganism are described above in this example.

Plant chitinases are potent growth inhibitors of fungi (Schlumbaum, A. et al., Nature 324:365-367 (1986)). These enzymes can be obtained from leaves of bean plants.

These antifungal proteins have also been identified in radish seeds (Terras, F. R. G. et al., J. Biol. Chem. 267:15301-15309 (1992)). Antimicrobial peptides have been isolated from the seeds of the four o'clock plant (Mirabilis jalapa L.) (Cammue, B.P.A. et al., J. Biol. Chem. 267:2228-2233 (1992)). Other plant-derived enzymes useful as crop protection agents include lysozyme and 1,3-glucanases.

Crop protection agents may also be obtained from non-plant sources, such as vertebrates and invertebrates. Zasloff has identified a class of antimicrobial peptides from vertebrates, notably frog skin, referred to as magainins (Zasloff, M., Proc. Natl. Acad. Sci. 84:5449-5453 (1987); Bevins, C. L. and Zasloff, M., Ann. Rev. Biochem. 59:395-414 (1990)). These peptides each contain 23 amino acids, and at low concentrations they inhibit the growth of numerous

species of bacteria and fungi, and induce osmotic lysis of protozoa. The cDNAs of magainin peptides have been cloned, and additional magainin-type molecules have been identified and characterized. Another class of proteins having strong anti-bacterial activity is the family of cecropins. Cecropin molecules have been identified in many species of insects, which are invertebrates. The amino acid sequences for major cecropins are known and have been published (Boman, H. G. et al., Ann. Rev. Microbiol. 41:103-126 (1987)). A class of anti- bacterial proteins having a larger molecular weight than cecropins has been identified, and these proteins are referred to as attacins (Boman et al.). Attacins have been identified in moth species.

An additional approach is to enhance the activity of pesticidal proteins by use of protease inhibitors. Serine protease inhibitors have been shown to enhance the activity of delta-endotoxin proteins of Bacillus thuringiensis against several important agricultural pests (MacIntosh, S.C. et al., J. Agricultural and Food Chemistry 38:1145-1152 (1990)). Trypsin inhibitors from cowpea and other legumes are shown to be effective antimetabolites active against, for example, a beetle (Angharad, M.R. et al., J. Sci. Food Agric. 34:345-350 (1983)). The synthesis of defensive proteins in plant tissues can be induced by a proteinase inhibitor (Pearce, G. et al., Science 253:895-898 (1991)).

Additional agricultural chemical producing genes useful in the present invention are those that encode enzymes active in the synthesis of secondary metabolites. One such class includes the polyketides and structurally related compounds such as actinorhodin. Genes encoding the actinorhodin synthetic pathway can be inserted into endophytes for the control of plant fungal diseases, including rice blast. Genes encoding enzymes for the synthesis of polyketide antibiotics can be obtained from microorganisms.

However, microorganisms are not the only source of genes for the production of agriculturally useful secondary metabolites having antibiotic activity. The steroidal

compound squalamine, found in sharks, also demonstrates an activity against E. coli, Staphlococcus, and Streptococcus. See, Stone, R. in Science 259:1125 (1993). Anti-microbial chemicals, termed phytoalexins, are produced by a wide specturm of higher plants in response to infectious agents. See, Robert N. Goodman, Zoltan Chiraly and K.R. Wood, in The Biochemistry and Physiology Of Plant Disease, (U. Missouri Press, Columbia, MO, 1986).

This example therefore also describes methods for expressing genes encoding enzymes that synthesize secondary metabolites in recombinant bacteria. The general methods for preparing an integration vector and transfecting a microorganism are described above in this example.

The molecular cloning of the whole biosynthetic pathway of the Streptomyces antibiotic actinorhodin was first described by Malpartida, F. et al., Nature 309:462-464 (1984). Malpartida et al. demonstrated that the genes encoding the full biosynthetic pathway for actinorhodin can be transferred between bacteria. A continuous segment of Streptomyces coelicolor DNA carrying the complete genetic information required for actinorhodin synthesis was introduced into Streptomyces parvulus, and actinorhodin was synthesized by the recombinant host.

Actinorhodin is synthesized from acetate units through the polyketide pathway. Other polyketide antibiotics are produced by fungi, actinomycetes, mycobacteria, and other bacteria. (See, Hopwood, D. A. et al., Ann. Rev. Genet. 24:37-66 (1990).

In addition to naturally-occurring antibiotics, the methods described herein are useful for producing hybrid agricultural-chemical-producing microorganisms capable of synthesizing novel hybrid antibiotics. Such methods are known in the art, as exemplified by Hopwood, D. A. et al., Nature 314:642-644 (1985). Briefly, as a non-limiting example, a plasmid containing the genes for the synthesis of an antibiotic, such as actinorhodin, is inserted into a microorganism producing a different but preferably

structurally related antibiotic, such as medermycin or granacitin. Production of novel antibiotics can be detected, for example, as alterations in the acid-base characteristics of the culture relative to that of the wild-type organisms. Routine screening of the novel compounds for antibiotic activity will indicate recombinant hybrid antibiotic-producing microorganisms that are useful for hybridization with an infecting microorganism according to the methods of this invention.

Genes from any prokaryotic or eukaryotic source encoding any agricultural chemicals, or catalysts producing agricultural chemicals, can be cloned into the Cxc integration vector as described above in the example of Bacillus thuringiensis endotoxin. It should be understood by a person skilled in the art that agricultural chemicals can include nucleic acids, proteins, lipids and carbohydrate molecules, and their precursors. Stable transformants are then screened for the production of the agricultural chemical products.

EXAMPLE 6

CONSTRUCTION OF A CXC/BT MICROBE

This example sets forth (1) the construction a Cxc/Bt microbe and (2) a verification of that structure. The test microbe consists of a Maryland strain of Clavibacter xyli subsp. cynodontis ("Cxc") that has been genetically engineered to produce the delta endotoxin of Bacillus thuringiensis subsp. kurstaki ("Bt") and has been designated as MDR1.586.

MATERIALS AND METHODS

1. Test Microbe

   A. Construction of the Cxc plasmid integration vectors. The integration plasmid used to form MDR1.586 was pCG741.

      i. Materials.

The DNA segments comprising the integration vectors were obtained as follows:

1) The E. coli replicon

The E. coli replicon pGEM 5Z (f+) was acquired commercially from Promega (Madison, WI). The colE1 origin of replication of this plasmid did not function in Cxc, nor did the ampicillin resistance gene.

2) The homologous Cxc sequence

A chromosomal DNA fragment was isolated from Cxc which served as a site for homologous recombination, and which also provided a source for the promotion of mRNA synthesis in the vector, pCG741. The fragment was generated by the cloning of Sau3a fragments into the E. coli replicon pUC19 to produce a Cxc library.

3) The delta-endotoxin gene

The delta-endotoxin gene of B. thuringiensis subsp. kurstaki HD73 was acquired from Dr. Arthur Aronson of Purdue University. The gene was cloned as a HinDIII fragment in an E. coli cloning vector. The complete sequence of the gene and flanking DNA is given in Adang, M.J.K., M.J. Staver, T.A. Rocheleau, J. Leighton, R.F. Barker and D.V. Thompson, 1985, Characterized full-length and truncated plasmid clones of the crystal protein of Bacillus thuringiensis subsp. kurstaki HD-73 and their toxicity to Manduca sexta, Gene 36:289-300. An abbreviated restriction map of the gene and flanking sequence is shown in Fig. 6. A portion of the sequence 5' to the N-terminus of the gene was modified by CGI with synthetic DNA. The relevant portion of the altered and native sequence are shown in Fig. 12. Functionally, CGI removed the Bt promoter region 5' to the natural leader transcription site, introduced several translation

stop codons, and generated a polylinker region for directed DNA fusions.

4) The tetracycline resistance marker

A 4.99 kb DNA fragment containing a tetracycline resistance determinant was obtained from the conjugative plasmid Tn916. This determinant has been designated tetM (Burdett, V; J. Inamine, S. Rajagopalan, 1982, Heterogeneity of tetracycline resistance determinants in streptococcus J. Bacteriol 149:995-1004) and operates at the level of protein synthesis (Burdett, V., 1987, Streptococcal tetracycline resistance mediated at the level of protein synthesis, J. Bacteriol 165:564-569). Homologues of this determinant, obtained from the transposon Tn1545 (Martin, P., P. Trieu-Cuot and P. Courvalin, 1986, Nucleotide sequence of the tetM tetracycline resistance determinant of the streptococcal conjugative shuttle transposon Tn1545 Nuc. Acids Res. 14) and from Ureaplasma urealyticum (Sanchez-Pescador, R., J.T. Brown, M. Roberts, and M.S. Urdea, The nucleotide sequence of the tetracycline resistance determinant tetM from Ureaplasma urealyticum, Nuc. Acids Res. 16) have been sequenced and shown to be 95% homologous at the amino acid level.

Analysis of the tetM gene of Tn916 shows it to be highly homologous to other sequenced tetM genes, and no functionality associated with transposition was found in this sequence.

The 16.4 kb transposon Tn916 was originally discovered on the chromosome of Enterococcus (Streptococcus) faecalis DS16 (Franke, A.E., and D.B. Clewell, 1981, Evidence for a chromosome-borne resistance transposon (Tn916) in Streptococcus faccalis that is capable of "conjugal" transfer in the absence of a conjugative plasmid J. Bacteriol. 145:494-502).

The E. coli plasmid pAM120 was constructed by cloning a fragment of the Enterococcus plasmid pAD1 containing Tn916 into a pBR322 derived vector (Clewell, D.B. and C. Gawron-Burke, 1986, Conjugative transposons and the dissemination of antibiotic resistance in Streptococci, Ann. Rev. Microbiol. 40:635-659). The tetM gene has been previously mapped to a 4.9 kb HincII fragment (Fig. 8) within the transposon (Clewell, et al. 1986).

We used the 4.9 kb HincII tetM fragment (Fig. 3) for integration vector construction. This fragment, although derived from a conjugative transposon, is unlikely to possess the capacity for mobilization through either transposition or conjugation. This conclusion has been reached by a consideration of the sequence and by literature in which the functions of excision, transposition, and conjugal transmission have been genetically analyzed.

The conjugative transposon Tn916 is able to transpose to plasmids (e.g., pAD1 in E. faecalis, above) and to transfer to the chromosomes of strains of other Streptococcal species by conjugative functions encoded within the transposon. By "hitchhiking" on other conjugative plasmids and by conjugation, Tn916 has also been shown to transfer to other genera of bacteria, including some gram negatives (Clewell et al. 1986).

Senghas, et al. (Senghas, E., J.M. Jones, M. Yamamoto, C. Gawron-Burke, and D.B. Clewell, 1988, Genetic organization of the bacterial conjugative transposon Tn916, J. Bacteriol 170:245-249) carried out a study in which various functions Tn916 were insertionally inactivated by Tn5 mutagenisis. The result of this work was that the functions of excision, transposition and conjugative transfer

via transposition were mapped to portions of the transposon outside of the HincII fragment containing the tetM gene. The majority of the critical functions for conjugation mapped to the right of the HincII tetM fragment (Figure 9). Significantly, three insertional mutations inactivating the excision function, which mapped at the left end of Tn916 outside the tetM fragment, could be complemented by the separately cloned end showing the dissociation of this function from tetM.

Additional evidence for the functions localized at the left end of Tn916 comes from the genetic analysis of Tn1545. This is also a conjugative transposon containing tetM, and shows virtual identity to Tn916 at its extremities, which are the sites of transpositional insertion (Caillaud, F., and P. Courvalin, 1987, Nucleotide sequence of the ends of the conjugative shuttle transposon Tn154, Mol. Gen. Genet. 209:110-115; Clewell, D.B., S.E. Flannagan, Y. Ike, J.M. Jones, and C. Gawron-Burke, 1988, Sequence analysis of termini of conjugative transposon Tn916 J. Bacteriol. 170:3046-3052). Poyart-Salmeron C., P. Trieu-Cuot, C. Carlier, and P. Courvalin, 1989, Molecular characterization of two proteins involved in the excision of the conjugative transposon Tn1545 homologies with other site-specific recombinases EMBO (Eur. Mol. Biol. Organ) 8:2425-2434 have determined the sequence of the end of Tn1545 corresponding to the left end of Tn916. Here they found two open reading frames (ORF1 and 2) with substantial (35%) homology to the carboxy half of the Pin protein of E. coli which functions in site-specific recombination; these ORF's corresponded to two proteins made in a cell-free system. These functions were able to complement,

in vivo, mutations which inactivated transposition of Tn1545

These data have led to a model of transposition of Tn916 and Tn1545 which predicts that excision of the transposon is the first and rate-limiting step of transposition, that the ORF2 protein is essential for that process, and that a circular intermediate results which can then insert into other sites (Clewell et al. 1986 and Poyart-salmerson et al. 1989). Just such an intermediate of Tn916 has been detected and purified, (Scoot, J. R. P.A. Kircchman, and M.B. Caparon, 1988, An intermediate in transposition of the conjugative tranposon Tn916, Proc. Natl. Acad. Sci. 85:4809-4813) and is able to transpose when introduced into B. subtilis.

The HincII fragment containing the tetM gene of Tn916 lacks both essential ends of the transposon, including the ORF1 and 2 regions, plus other critical functions to conjugation mapped by Tn5 mutagenesis outside of this fragment. There is, therefore, no basis to expect that this fragment can excise, promote conjugation or transpose itself or any element associated with it.

5) Other DNA Segments

Several "polylinkers" of synthetic DNA were employed to specifically join segments of the integration vectors (see below).

ii. Construction Sequence.

1. Synthesis of pCG563, the basic integration vector.

Fig. 10 illustrates the generation of pCG563. The 4.8 kb tetM fragment was first cloned into pGEMSZ(f+) to produce the intermediate plasmid pCG560. The integration sequence from pCG219 was cloned into pCG560 to produce pCG563. This vector was capable of

transforming MDE1 to Tcr when plated on 2 sg tetracycline/ml of plate medium.

2. Modification of the CryIA crystal protein gene.

Fig. 4 illustrates the cloning and Fig. 12 the modification of the crystal protein gene of B. thuringiensis kurstaki HD73. A portion of Bt DNA was cloned in two steps from the original vector FL15 to give the intermediate vector pCG910. The fragment was reduced in size by cloning the 3.77 kb NdeI fragment into pGEMSZ(f+), to give pCG737. At this point a synthetic polylinker was added at the BstXI site to generate pCG738.

Removal of sequence 5' and 3' to the NdeI sites has the following consequences: the B. thuringiensis promoters I and II are removed from the 5'end, and the second of two inverted repeat stem-loops 3' to the gene is removed. The addition of the polylinker creates promoter cloning sites at the 5'end, as well as translation stop codons. A comparison of native and artificial DNA sequence at the 5' end is shown in Fig. 12. All native Bt sequence 5' to the NdeI site has been removed and substituted, as shown.

3. Construction of pCG741.

Fig. 13 shows the construction of the Bt integration plasmid pCG741.

The plasmid pCG741, when transformed into Cxc, produces the 130 kd toxin, plus degradation products of the protein (see Example 10). The source of promotion for expression has been determined by S1 nuclease mapping to be the Cxc DNA 5' to the ApaI site if the Bt polylinker.

B. Transformation of Cxc strain MDE1 with pCG741.

i. Materials

1) Cxc strain MDE1.

Strain MDE1 is the wild-type Maryland isolate.

2) DNA.

Plasmid DNA was prepared from E. coli by equilibrium density centrifugation according to standard techniques (Maniatis et al.)

ii. Methods

1) Preparation of cell samples.

Cultures of MDE1 were grown in S27 medium at 30°C to late-log phase. The cells were harvested by centrifugation in the cold (4°C) and washed with ice-cold water. The final cell suspension was brought up in 10% sucrose to a concentration of ca. 1010 cells/40 ul.

2) Electroporation.

Plasmid DNA was added to 40 ul samples of the cell suspension and transferred to a prechilled electroporation cell (Bio-Rad). Electroporation took place in the Bio-Rad Gene Pulser. The cells were then diluted with S27 medium at 22°C and plated on cellulose acetate filters atop SC plates. After 18 hr at 30°C the filters were transferred to SC plates containing 2 ug tetrlcycline/ml.

3) Growth of transformants.

After approximately 10 days Tcr colonies appeared. The transformants were purified and their Bt expression determined. A representative Cxc transformant containing pCG741 was designated MDR1.586.

2. Test Microbe Characteristics

A suspension of Cxc strain MDR1.586 or MDE1 in water is a yellow odorless liquid, density approximating water. The pH is approximately 6. The cells are 0.2-0.3 u in diameter and 1-2 ul in length. A typical suspension contains 10^9-10^{10} per ml. The cells, when frozen at -20°C, are stable for at

least one month, or a year if glycerol is added to a final concentration of 50%.

3. Method for Verifying Integration

Plasmid pCG741 integrated into the Cxc chromosome by crossover within the region of DNA homology defined by the Cxc chromosomal fragment. This recombination converted the circular plasmid to a linear form within the Cxc chromosome. To detect this conversion of the circular plasmid to a linear insert, the technique of Southern hybridization was used to map the borders of the integrated DNA. The overall structural integrity of the insert was determined by comparing restriction fragment mobilities of the inserted DNA with that of the pure plasmid. The presence of border fragments - fragments with one restriction site within the plasmid and the other site within the Cxc genome beyond the region of homology - was used to show both the fact of chromosomal integration as well as the site of integration.

4. Test System

The test system used for this study was restriction site analysis of DNA isolated from the test microbe, MDR1.586.

ANALYTICAL METHODS

Total DNA from MDR1.586 was purified and cut with specific restriction endonucleases. DNA fragments were separated by agarose gel electrophoresis, and the pattern of restriction fragments was determined by hybridization with specific radiolabelled DNA probes:

a. Source of Probes (Fig. 14).

Bt gene: the 600 bp EcoRI fragment.

pGEM 5Z (f+): the complete 3 kb plasmid.

TetM: the 1.7 kb Xbal - HindIII fragment.

Cxc integration sequence: the 5kb Xbal-EcoR1 fragment.

b. Restriction Digests.

In order to determine whether integration occurred, the restriction enzyme SfiI was used which cut pCG741 once in a non-Cxc portion of the plasmid (Fig. 15). Cleavage at this site allows an

unintegrated plasmid to be detected as the linear fragment. If the plasmid is integrated, the plasmid Sfil site would generate two new fragments from Sfil sites in the genome outside the plasmid boundaries (Fig. 15).

In order to assess the integrity of the integrated plasmid, the restriction enzyme Kpn1 was chosen. This enzyme has four sites in pCG741, one of which is in the Cxc portion of the plasmid (Fig. 15), and the fragments generated correspond to the four different elements of the plasmid. If the plasmid integrates by a Campbell-type crossover event which generates a duplication of the Cxc integration sequence (Fig. 15), Kpn1 will generate a circularly permuted map of the integrated plasmid DNA indistinguishable from the unintegrated plasmid. This digest generates the fragments shown in Fig. 15.

The patterns were visualized by autoradiography and their mobilities determined by reference to DNA molecular weight standards.

1. Isolation of DNA.

DNA was isolated from Cxc strains MDE1 and MDR1.586 by adaptations of the standard cleared-lysate procedure (Maniatis, T., E.F. Fritsch and J. Sambrook, 1982, Molecular Cloning, a Laboratory Manual, Cold Spring Laboratory, N.Y. (hereinafter "Maniatis, et al., 1982")). Lysozyme treated cells were disrupted with sodium dodecyl sulfate (SDS) and treated with proteinase K. After chloroform/phenol extractions the DNA was repeatedly precipitated from ethanol, and resuspended in TE buffer (0.01 M Tris-HCl, pH 8.0, 0.005 M Na-EDTA).

2. Restriction Enzyme Analysis.

DNA samples were digested with the indicated restriction enzymes according to manufacturers' instructions.

The digested samples were electrophoresed in agarose gels of indicated concentration in TAE buffer (Maniatis,

et. al., 1982) for lengths of time appropriate to resolve fragments of the expected size.

3. Transfer.

Following ethidium bromide staining and photography, the DNA fragments were denatured with base, neutralized, and transferred from the gels to "Gene-Screen Plus" membranes (New England Nuclear Research Products) according to the manufacturer's instructions. The transfer was done using a vacuum apparatus (Vacu-Blot, ABN products) according to manufacturer's instructions.

4. Hybridization.

Hybridization probes were prepared from the indicated restriction fragments resolved on agarose gels by standard techniques (Maniatis, et al., 1982). The fragments were labeled with 32P-alpha dCTP by the "oligo labelling" technique with the appropriate kit (Boehringer).

Hybridization of the labeled probe with the membrane-bound , DNA fragments was carried out according to instructions provided by the manufacturer of the membrane (New England Nuclear). The gels were hybridized with the indicated probes in a solution containing 1 M NaCl, 1% SDS (sodium dodecyl sulfate) and 10% dextran sulfate at 65°C for 18 hrs. The membranes were washed twice with (a) 2 x SSC (0.3 M NaCl, 0.03 M Na Citrate) for 5 min, 21 C; (b) 2 x SSC plus 1% SDS at 65 C for 30 min; (c) 0.1 x SSC at 21 C for 30 min. The membranes were autoradiographed at 22 C for varying lengths of time to provide a clear image.

Mobilities of fragments were determined by reference to stained molecular weight standards.

RESULTS

Autoradiographic data from Southern hybridizations:

Fig. 16 shows the result of SfiI digests of DNA samples, specifically probing for Cxc DNA homologous to the integration sequence. Lane 5 (strain MDE1 DNA) shows a 16.2 kb hybridizing fragment, corresponding to the region of the native genome which contains the DNA of the integration

vector. Lane 1 shows the uncut, and lane 2 the SfiI digested plasmid pCG741 alone. The latter 17 kb fragment corresponds to the position of linear plasmid. Lane 3 shows the digest of an artificial mixture of pCG741 and MDE1 DNA. The native Cxc SfiI fragment and the linear plasmid migrated similarly, and form a doublet. Lane 4 shows the SfiI digest of strain MDR1.586. Both the native genome fragment and the linear plasmid band are missing, replaced by two new fragments (22.8 and 11.7 kb) which sum (34.5 kb) reflects the sum of the two they replaced (33.2). These data show that the plasmid has been converted to a linear form integrated into the chromosome of Cxc, at the site of homology corresponding to the integration sequence of the plasmid.

Fig. 17 shows the result of KpnI digests of DNA probed with probes homologous to different portions of the integration plasmid pCG741. Each subset is the identical digest probed with a different probe. The data show that the integrated plasmid in strain MDR1.586 (lane 4) gives the same bands as an artificial mixture (lane 3) of the pure plasmid (lane 2) and MDE1 DNA (lane 5); these data show that the integrated plasmid has not been rearranged or deleted, within the sensitivity of the technique.

The amount of plasmid DNA (5 ng) in the artificial mixture of MDE1 (2 ug) and plasmid was computed to be equal to a frequency of one copy per genome (ca. 4000 kb). The similar hybridization of the plasmid signal to that of the homologous DNA fragment in the recombinant MDR1.586 shows that there is approximately one integrated plasmid per genome.

CONCLUSIONS

The results of the Southern analysis demonstrated that:

1. The restriction fragment map of pCG741 and the identity of the different components of the integrated plasmid in MDE1 chromosome were the same. The plasmid did not undergo detectable rearrangements following integration.

2. A restriction enzyme digest fragment of the wild-type Cxc chromosome (MDE1), the target site of integration, disappeared as expected in the recombinants. This demonstrated that its linear continuity had been altered. Two new fragments appeared in the recombinant, corresponding to the two border fragments expected at the junction of the integrated linear plasmid and the target site on the Cxc chromosome. Integration relied on homologous recombination between the cloned segment of chromosomal DNA in pCG741 and the chromosomal site in MDE1. The plasmid was inserted in its entirety with no detectable rearrangements.

3. No unintegrated plasmid was detected. Plasmid CG741 does not exist in an unintegrated form in Cxc.

4. The autoradiographic intensity of the hybridized integrated sequences was equivalent to the expected intensity of a single copy of the relevant sequence diluted in the Cxc genome. The copy number of the integrated plasmid is similar to that of the genome.

EXAMPLE 7

IN PLANTA LABORATORY AND GREENHOUSE BIOASSAYS OF CXC/BT CONSTRUCTIONS AGAINST EUROPEAN CORN BORER

The objective of this study is to determine the ability of a Cxc/Bt recombinant strain to prevent or reduce damage to corn caused by artificial infestations of the European corn borer (ECB), Ostrinia nubilalis (Hubner), in the greenhouse.

MATERIALS/METHODS

Test Microbe

The test microbe used in the experiments reported here was Crop Genetics strain MDR1.586 (genotype pCG741/MDE1; phenotype TetR SC+Tet; prepared as in Example 6 above). The wild-type Maryland isolate of Cxc (MDE1) was used as an endophyte control. Cells were stored in 50% glycerol at -20xC or in 2% BSA at -80xC. Viability checks were performed on both strains prior to use in each experiment (Table 3).

Test System

The test system for this study is field corn. The European corn borer was chosen as the test insect because it is the primary target pest for Cxc/Bt.

Test Site

Plant lot IP89-3 (field corn variety PD 003) was planted July 20, 1989, and inoculated August 12 with either Cxc/Bt recombinant or wild-type Cxc suspended in PBS, or with sterile PBS, using the stab inoculation method. Plant lot IP89-4 (also variety PD 003) was planted August 17 and inoculated August 30. Plant lot IP89-5 (varieties PD 003 and PD 093) was planted October 6 and inoculated October 23. The strain numbers for test microbes used in these experiments are presented in Table 3.

Each experiment was arranged in a split-plot design with individual greenhouse benches as main plots and treatment groups as sub-plots arranged in rows, the positions of which were randomized within each bench. Experiment IP89-3/GH-1 occupied 3 benches, each bench containing 7 plants per treatment group for a total of 21 plants per treatment. Experiment IP89-4/GH-1 also occupied 3 benches, but each treatment was represented by 30 plants (10 per bench). Experiment IP89-5/GH-1 occupied 4 benches, with 24 plants per treatment (6 per bench) for each of 2 corn varieties (PD 003 and PD 093). Experiment IP89-5/GH-2 also occupied 4 benches,

with 24 plants per treatment (6 per bench) for both corn varieties.

Inoculation

Inoculating devices were prepared by sharpening the eye end of No. 18 tapestry sewing needles. Needle volume was determined gravimetrically by the amount of water held in the eye. Only needles which held 3 ul (+/- 10%) were used. The needles were mounted in chucked metal handles, and the whole apparatus autoclaved before use. Inoculum was introduced into the plants at approximately 10 - 20 cm above the soil line by dipping the needle in a suspension of the test microbe or wild-type Cxc to fill the eye, then stabbing with the needle eye through to the center of the stem. The needle was then carefully withdrawn to ensure that the inoculum had been retained in the plant. Each plant was stabbed twice from different angles to insure it received sufficient inoculum. The dose was calculated to be between approximately 5 x 106 and 8 x 107 CFU per plant (Table 3). Procedures for inoculating plants are provided in co-pending United States Patent Application Serial No. 07/368,167, filed June 6, 1989, to Jed Fahey, entitled "Delivery of Beneficial Microorganisms to Seeds and Plants."

Test Method

Only plants confirmed as colonized by Cxc or Cxc/Bt (except PBS controls) were infested with insects. Details of the assessment of colonization incidence are presented below under "Phase Contrast Microscopy." The numbers of plants given above for each experiment (see "Test Site") include only colonized, infested plants.

Plants were infested with European corn borers 5 to 6 weeks after inoculation, depending on incidence of colonization. Experiment IP89-3/GH-1 was infested on September 18, 37 days after inoculation. Experiment IP89-4/GH-1 was infested October 16, 47 days after inoculation. Experiment IP89-5/GH-1 was infested December 4, 42 days after inoculation. Experiment IP89-5/GH-2 was infested December 5, 43 days after inoculation.

Experiments IP89-3/GH-1, IP89-4/GH-1, and IP89-5/GH-1 were infested by drilling a 1/4-inch hole about 3/4 of the way through the stalk in 5 internodes per plant, beginning with the lowest internode that was easily accessible. The drill bit was surface disinfested between treatment groups by dipping it in 95% ethanol and flaming. Each drill hole was infested with 3 neonate corn borer larvae from egg masses obtained from French Agricultural Research Service (Lamberton, MN). Egg masses were held at 10xC in a Percival chamber for 7 days to synchronize development, then transferred to another Percival chamber at 27xC for approximately 48 hours for incubation and eclosion. Larvae were transferred into the stem holes using camel hair brushes, and the holes were then plugged with nonabsorbent cotton. Experiment IP89-5/GH-2 was infested by placing 10 neonate ECB larvae into each of 5 leaf axils per plant using a camel hair brush. This technique provided a more realistic simulation of a natural infestation. The source and storage conditions of egg masses were the same as in the other experiments.

Sampling/Observations

All plants were assessed for damage due to corn borer feeding 3 to 4 weeks after infestation. The dates for each experiment were: IP89-3/GH-1, October 9 and 10; IP89-4/GH-1, November 6 and 7; IP89-5/GH-1, January 2, 1990; IP89-5/GH-2, January 3 and 4, 1990). Each plant was cut down at the soil line and stripped of its leaves, which were examined for evidence of larval feeding. The stalk was then split longitudinally to expose corn borer feeding tunnels and any larvae present. Ears were also dissected to expose tunnels and insects. Data recorded for each plant included the length of each tunnel, the weight and developmental stage (instar) of each live insect recovered, and the instar of any dead insects found in or on the plant.

ANALYTICAL METHODS

Description of Viability Procedure

Inoculum for greenhouse experiments was prepared from 5-to 8-day-old cultures of Cxc or Cxc/Bt grown on solid media (SC for Cxc isolate MDE1, SC + Tet2 for Cxc/Bt strains MDR1.586). Cell mass was collected from the surface of the medium with a sterilized rubber policeman and suspended in PBS to a weight ratio of approximately 0.05g per ml. The resulting concentration of viable cells was between 109 and 1011 CFU/ml when diluted and plated on solid media for verification (see Table 3).

Calculations

Tunnel lengths were summed to yield total tunnel length for each plant, providing an estimate of the total amount of damage caused by corn borer feeding. Weights of surviving insects were summed to yield total insect biomass per plant, a composite measure that incorporates both survival and growth of the insects.

Phase Contrast Microscopy

Phase contrast microscopy was used to determine incidence of colonization of plants inoculated with Cxc and Cxc/Bt strains. The lowest leaf not showing an inoculation scar was removed from each plant, and sap was expressed from the midrib onto a microscope slide. The drop of sap was examined under oil immersion at 1000X. The plant was scored as positive if at least 2 Cxc cells were observed within 30 seconds of viewing a single field. If fewer than 2 Cxc cells were observed, or if there was uncertainty about the sample, the plant was scored as negative. The selection criterion of 2 cells per field corresponds to approximately 107 CFU/ml sap.

Colonization incidence sampling began at least 4 weeks after inoculation, and if necessary it was repeated weekly thereafter (up to 6 weeks after inoculation) until at least 20 plants in each inoculation treatment group were confirmed colonized.

Statistical Methods

For Experiments IP89-3/GH-1, IP89-4/GH-1, and IP89-5/GH1, number of tunnels and total tunnel length per plant, number of live insects recovered per plant, and the total corn borer biomass recovered per plant (the sum of individual weights of insects from each plant) were analyzed by split-plot analysis of variance (alpha = 0.05), with benches as main plots and treatment groups as subplots. Treatment means were separated using Duncan's multiple range test at alpha = 0.05. For Experiment IP89-5/GH-2, these data were analyzed by split-split-plot ANOVA (alpha = 0.05), with benches as mainplots, corn hybrids as subplots, and treatment groups as sub-subplots. For all experiments, unbalanced data (average tunnel length, average weights and instars of live insects) were summarized as means and their standard errors, but were not subjected to ANOVA. All analyses and calculations of descriptive statistics were performed using the MSTAT statistical software (Nissen, O., E.H. Everson, S.P. Eisensmith, V. Smail, J. Anderson, K. Rorick, G. Portice, D. Rittersdorf, P. Wolberg, M. Weber, R. Freed, B. Bricker, T. Heath, and J. Tohme. 1985. User's guide to MSTAT (version 4.0). Michigan State University, East Lansing)

RESULTS/DISCUSSION

Inoculation of corn plants with Cxc/Bt resulted in significant reduction in feeding damage caused by European corn borer larvae. In most cases, significant effects of Cxc/Bt on corn borer survival, growth, and development were also observed.

Experiment IP89-3/GH-1

The number of borer tunnels in plants inoculated with Cxc/Bt strain MDR1.586 was reduced to one third of the number of tunnels found in either control group, while total tunnel length was reduced by more than 70% (Table 4).

Only 14 to 15 percent of the larvae placed into the stalks of control plants were recovered alive at the end of the experiment. High larval mortality is common in European corn borer populations (Showers, W.B., and G.L. Reed, 1972,

methods of infesting with corn borer, Iowa State J. Sci. 46:429-434; Showers, W.B., M.B. DeRozari, G.L. Reed, and R.H. Shaw, 1978, Temperature-related climatic effects on survivorship of the European corn borer, Environ. Entomol. 7:717-723; Showers, W.B., J.F. Witkowski, C.E. Mason, D.D. Calvin, R.A. Higgins, and G.P. Dively, 1989, European corn borer: development and management, N. Central Regional Extension Publ. No. 327. Iowa State Univ., Ames, Iowa.; Siegel, J.P., J.V. Maddox, and W.G. Ruesink, 1987, Survivorship of the European corn borer, Ostrinia nubilalis (Hubner) (Lepidoptera: Pyralidaee in Central Illinois, Environ. Entomol. 16:1071-1075) even when larvae are placed directly into the relatively protective and nutritious environment of the stem (Chiang, H.C., 1959, Survival of European corn borer larvae, Pyrausta nubilalis (Hubn.), in artificial tunnels in stalks of field corn, Zea mays, L. Ann. Entomol. Soc. Am. 52: 631-632). By comparison, only about 6% of the larvae placed in plants inoculated with Cxc/Bt strain MDR1.586 were recovered alive at the end of the experiment. Total corn borer biomass was reduced by nearly 2/3 (relative to controls) in plants inoculated with Cxc/ Bt.

Experiment IP89-4/GH-1

Cxc/Bt strain MDR1.586 caused significant reduction in both the number of corn borer tunnels per plant and the total amount of feeding (total tunnel length) (Table 6). Significantly fewer live insects were recovered from Cxc/Bt-inoculated plants than from controls (a reduction of about 75%). Total corn borer biomass and average weight were also less for insects recovered from the Cxc/Bt-inoculated plants (Table 7).

Measurements of both plant damage and insect survival and growth were lower than those obtained previously, probably due to environmental changes (lower temperatures and changing photoperiod) associated with the changing seasons. Although the greenhouse was equipped with heaters and supplemental lighting, it was impossible to duplicate during the late fall and winter the conditions of

high temperature and relative humidity (as well as maximal solar illumination) that existed when the study began in the summer. As a result, insect feeding and growth rates, which are influenced strongly by environmental conditions (particularly temperature), tended to be reduced during the winter months.

Experiment IP89-5/GH-1

Damage due to corn borer feeding was significantly less in plants inoculated with Cxc/Bt recombinant strain MDR1.586 than in plants inoculated with wild-type Cxc or sterile buffer. The magnitude of this effect was greater in corn hybrid PD 003 than in hybrid PD 093. In PD 093, total tunnel length was reduced by about 50%, number of tunnels by about 40%, and average tunnel length by about 15%, relative to controls. The corresponding approximate percent reductions for hybrid PD 003 were 80%, 55%, and 60% (Table 8). MDR1.586 caused a significant reduction in the number and weight of surviving insects, but the magnitude of this reduction was again greater for hybrid PD 003 than for hybrid PD 093 (85% vs. 60% reduction in number of live insects per plant and 80% vs. 30% reduction in average weight per insect, relative to controls) (Table 9). In both control groups (sham-inoculated and wild-type Cxc), plants of hybrid PD 003 tended to suffer more damage (total tunnel length) and produce more ECB biomass (i.e. more and larger insects) than did plants of hybrid PD 093. However, the two hybrids did not differ significantly (except for total tunnel length) when inoculated with MDR1.586 (Tables 8 and 9).

Experiment IP89-5/GH-2

Corn plants inoculated with Cxc/Bt recombinant strain MDR1.586 suffered less damage than did control plants, despite heavy infestation pressure from externally-applied corn borers. Cxc/Bt-inoculated plants contained fewer ECB tunnels and less internal damage (measured as total tunnel length) than did control plants (Table 10). As in previous experiments, fewer and smaller live insects were recovered from Cxc/Bt-inoculated plants than from controls (Table 11).

Plants of hybrid PD 003 contained fewer tunnels, less internal damage, and fewer, smaller larvae than plants of hybrid PD 093, irrespective of inoculation treatment. However, the relative effect of Cxc/Bt strain MDR1.586 on these measurements was similar for both hybrids (i.e., the main effects of hybrids and treatment were highly significant by ANOVA, but their interaction was not).

CONCLUSION

The results of these experiments demonstrate that Cxc/Bt strain MDR1.586 is capable of preventing or limiting damage to inoculated corn caused by European corn borer under conditions of artificial infestation in the greenhouse. Experiments IP89-3/GH-1, IP89-4/GH-1, and IP89-5/GH-1 demonstrated that MDR1.586 produces sufficient amounts of BT endotoxin to affect larvae feeding in the relatively protected but highly Cxc/Bt-colonized stem. Experiment IP89-5/GH-2 demonstrated that this activity is sufficient to reduce feeding, growth, survival, and overall damage even when insects were applied to external plant surfaces to simulate more realistically a natural infestation by second-generation ECB.

Table 3

Viability of test microbes used in study 40ENT.

Strain	Experiment:	log CFU per ml inoculum			log CFU/plant (approx.)		
		IP89-3	IP89-4	IP89-5	IP89-3	IP89-4	IP89-5
MDE1		10.36	10.31	9.34	7.8	7.8	6.8
MDR1.586		10.36	9.26	9.60	7.8	6.7	7.1

Table 4

Measurements of plant damage, Experiment IP89-3/GH1. Data are means and standard errors (N=21 plants per treatment). Corn variety was PD093.

Treatment	Description	Total tunnel length/plant (cm)	Number of tunnels/plant	Average tunnel length (cm)
PBS	Sham inoc. control	15.0 ± 2.5 a	3.7 ± 0.6 a	4.3 ± 0.4
MDE1	Wild-type Cxc control	13.1 ± 1.8 a	3.6 ± 0.6 a	3.8 ± 0.4
MDR1.586	Cxc/Bt recombinant	3.7 ± 1.1 b	1.3 ± 0.4 b	2.8 ± 0.3

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 5

Measurements of surviving European corn borers, Experiment IP89-3/GH-1. Data are means and standard errors. Corn variety was PD093.

Treatment	Description	Live insects recovered Total	%	No./plant	Total ECB biomass per plant (mg)	Average Wt. (mg)	Avg. Instar
PBS	Sham inoc. control	48	15.2	2.3 ± 0.4 a	127 ± 25 a	53 ± 6	4.4 ± 0.1
MDE1	Wild-type Cxc control	44	14.0	2.1 ± 0.3 a	141 ± 22 a	67 ± 6	4.8 ± 0.1
MDR1.586	Cxc/Bt recombinant	18	5.7	0.9 ± 0.3 b	36 ± 13 b	42 ± 7	4.4 ± 0.2

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 6

Measurements of plant damage, Experiment IP89-4/GH-1. Data are means and standard errors (N=30 plants per treatment). Corn variety was PD093.

Treatment	Description	Total tunnel length/plant (cm)	Number of tunnels/plant	Average tunnel length (cm)
PBS	Sham inoc. control	7.1 ± 1.0 a	2.5 ± 0.3 a	2.8 ± 0.2
MDE1	Wild-type Cxc control	8.6 ± 1.1 a	2.7 ± 0.3 a	3.2 ± 0.2
MDR1.586	Cxc/Bt recombinant	1.2 ± 0.4 b	0.8 ± 0.3 b	1.5 ± 0.1

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 7

Measurements of surviving European corn borers, Experiment IP89-4/GH-1. Data are means and standard errors. Corn variety was PD093.

Treatment	Description	Live insects recovered			Total ECB biomass per plant (mg)	Average Wt. (mg)	Avg. Instar
		Total	%	No./plant			
PBS	Sham inoc. control	47	10.4	1.6 ± 0.2 a	62 ± 12 a	40 ± 4	4.6 ± 0.1
MDE1	Wild-type Cxc control	50	11.1	1.7 ± 0.2 a	96 ± 17 b	57 ± 5	4.7 ± 0.1
MDR1.586	Cxc/Bt recombinant	12	2.7	0.4 ± 0.2 b	7 ± 4 c	18 ± 5	4.3 ± 0.2

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 8

Measurements of plant damage, Experiment IP89-5/GH-1. Data are means and standard errors (N=24 plants per treatment).

Corn Hybrid	Treatment	Description	Total tunnel length/plant (cm)	Number of tunnels/plant	Average tunnel length (cm)
PD093	PBS	Sham inoc. control	20.4 ± 1.7 a	6.7 ± 0.4 a	3.0 ± 0.2
	MDE1	Wild-type Cxc	16.4 ± 1.8 b	5.2 ± 0.4 b	3.1 ± 0.2
	MDR1.586	Cxc/Bt recombinant	9.3 ± 1.4 c	3.6 ± 0.5 c	2.6 ± 0.2
PD003	PBS	Sham inoc. control	24.2 ± 1.9 a	6.1 ± 0.5 ab	3.9 ± 0.2
	MDE1	Wild-type Cxc	23.6 ± 2.6 a	5.7 ± 0.5 ab	4.1 ± 0.2
	MDR1.586	Cxc/Bt recombinant	4.6 ± 0.6 d	2.7 ± 0.4 c	1.7 ± 0.2

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 9

Measurements of surviving European corn borers, Experiment IP89-5/GH-1.
Data are means and standard errors.

Corn Hybrid	Treatment	Description	Live insects recovered Total	%	No./plant		Total ECB biomass per plant (mg)		Average Wt. (mg)	Avg. Instar
PD093	PBs	Sham inoc. control	86	24.9	3.7 ± 0.3	b	296 ± 30	b	79 ± 4	5.1 ± 0.1
	MDE1	Wild-type Cxc	68	18.9	2.8 ± 0.3	c	216 ± 30	c	78 ± 4	5.1 ± 0.1
	MDR1.586	Cxc/Bt recombinant	28	7.8	1.2 ± 0.2	d	62 ± 14	d	57 ± 8	4.7 ± 0.1
PD003	PBs	Sham inoc. control	113	31.4	4.7 ± 0.4	a	419 ± 40	a	89 ± 3	5.0 ± 0.1
	MDE1	Wild-type Cxc	104	30.1	4.5 ± 0.5	a	429 ± 52	a	95 ± 4	5.2 ± 0.1
	MDR1.586	Cxc/Bt recombinant	18	5.0	0.7 ± 0.1	d	15 ± 5	d	20 ± 6	3.7 ± 0.1

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 10

Tunneling damage caused by European corn borers applied as neonates to leaf axils, Experiment IP89-5/GH-2. Data are means and standard errors from 24 plants per hybrid and treatment.

Corn Hybrid	Treatment	Description	Total tunnel length/plant (cm)	Number of tunnels/plant	Average tunnel length (cm)
PD093	PBS	Sham inoc. control	34.9 ± 3.5 a	10.9 ± 1.0 a	3.3 ± 0.2
	MDE1	Wild-type Cxc	34.8 ± 2.8 a	10.4 ± 0.8 a	3.4 ± 0.1
	MDR1.586	Cxc/Bt recombinant	18.4 ± 2.1 c	6.7 ± 0.6 b	2.7 ± 0.2
PD003	PBS	Sham inoc. control	25.8 ± 2.1 b	8.3 ± 0.7 b	3.2 ± 0.2
	MDE1	Wild-type Cxc	20.8 ± 2.1 bc	7.7 ± 0.6 b	2.6 ± 0.2
	MDR1.586	Cxc/Bt recombinant	9.5 ± 1.1 d	4.0 ± 0.4 c	2.5 ± 0.3

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 11

Measurements of surviving European corn borers, Experiment IP89-5/GH-2.
Data are means and standard errors.

Corn Hybrid	Treatment	Description	Live insects recovered Total	%	No./plant		Total ECB biomass per plant (mg)		Average Wt. (mg)	Avg. Instar
PD093	PBs	Sham inoc. control	207	17.3	8.6 ± 0.9	a	708 ± 80	a	82 ± 3	4.9 ± 0.03
	MDE1	Wild-type Cxc	195	16.0	8.1 ± 0.8	a	717 ± 75	a	88 ± 3	5.0 ± 0.03
	MDR1.586	Cxc/Bt recombinant	105	8.8	4.4 ± 0.4	c	281 ± 27	c	64 ± 4	4.8 ± 0.1
PD003	PBs	Sham inoc. control	174	14.5	7.3 ± 0.7	ab	532 ± 55	b	73 ± 3	4.8 ± 0.04
	MDE1	Wild-type Cxc	151	12.6	6.3 ± 0.6	b	498 ± 55	b	79 ± 4	4.9 ± 0.1
	MDR1.586	Cxc/Bt recombinant	68	5.7	2.8 ± 0.3	c	154 ± 22	c	54 ± 4	3.7 ± 0.1

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

EXAMPLE 8

HOST RANGE OF CXC/BT CONSTRUCT

This example compares the survival and systemic distribution of a new Cxc/Bt construction, set forth above in Example 6, with that of an older, extensively tested construction and wild-type Cxc in 6 different plant species. These species appear to serve as differential hosts, exhibiting a range of colonization levels.

MATERIALS AND METHODS

Test Microbes:

The first control strain utilized in this experiment, strain MDE1, is a typical wild-type Cxc isolate. It was cultured from a lot which had been stored as 50% glycerol stocks at -20°C. This strain shows characteristic colony and cell morphology when cultured on SC media and SCM media, no growth on NBY (nutrient broth yeast extract agar), and a positive reaction to anti-Cxx antisera.

The second control strain, MDR1.3, was derived from MDE1 and contains a Bt gene, a kanamycin resistance gene, and a tetracycline resistance gene. This strain was cultured from EPS lot 1 which had been stored as 50% glycerol stocks at -20°C. This strain shows characteristic colony and cell morphology when cultured on SC media and SCM media, no growth on NBY media, and a positive reaction to anti-Cxx antisera.

As set forth above in Example 6, the test strain, MDR1.586, was derived from MDE1 and contains a Bt gene and a gene for tetracycline resistance. This strain was cultured from a lot which had been stored as a 50% glycerol stock at -80°C. This strain shows characteristic colony and cell morphology when cultured on SC and SCM, no growth on NBY, and a positive reaction to anti-Cxx antisera.

Test System:

The test system used in this experiment consisted of six different plant species grown in 4" pots. Test species were (1) redroot pigweed (Amaranthus retroflexus), (2) velvetleaf (Abutilon theophrasti), (3) ivyleaf morningglory (Impomoea

hederaceae), (4) jimsonweed (Datura stramonium), (5) ragweed (Ambrosia artemisiifolia), and (6) chive (Allium schoenoprasum).

Inoculation:

Cells were scraped from the surface of 6-8 day old plate cultures and suspended in sterile phosphate-buffered saline at a ratio of 1 g cells:1000 ml PBS. This suspension was inoculated into the plants approximately 2-3 cm above the soil line. The inoculating device was the sharpened eye of a #7 hand sewing needle held in a handle. At inoculation, the morningglory was 19 days old, the pigweed and jimsonweed were 26 days old, the ragweed and velvetleaf were 33 days old, and the chive was 40 days old.

The calculated inoculum dose for control strain 1 (MDE1) was 1.9 x 106 CFU/g, for control strain 2 (MDR1.3) was 2.1 x 106 CFU/g, and for the test strain (MDR1.586) was 2.4 x 106 CFU/g.

Test Method:

Inoculated plants were randomly arranged on the greenhouse bench in a split-plot design with plant species as the main plots and strains as subplots. There were 6 plant species, 3 strains, and 10 replications.

Sampling:

Samples were collected over a 5 day period (2 replications per day) from 27 to 31 days post-inoculation. Plant parts assayed were: (1) a basal shoot section (including the inoculation wound site if visible), and (2) a distal sample taken from at least 10 cm above the basal sample. Distal samples from pigweed, morningglory, and chive were pooled from 3 branches from the equivalent region. Samples were assayed on the day of collection.

ANALYTICAL METHODS

Inoculum Viability Procedure:

The inoculum suspension was serially diluted in phosphate-buffered saline, and 0.1 ml droplets of the appropriate dilutions were placed on SC medium. Counts were made after 8 days incubation at 28 + 3C in the dark. The

dilution which gave counts the closest to between 8 and 80 colonies per droplet was counted.

Culture Method:

1. Disinfestation

a. Samples were washed of any adhering dirt with a weak detergent solution.

b. Samples were surface disinfested by immersion (with some agitation) in a solution of 10% bleach and 1 drop/ 1 Tween 80 for two minutes.

c. Samples were rinsed twice by immersion in sterile distilled water changed at appropriate intervals, and blotted dry on sterile paper towels. A minimum of about 5 mm of tissue was aseptically removed from the cut ends to eliminate any disinfestant which may have been absorbed by the tissue.

2. Plating

a. Thin slices of the sample were aseptically weighed and adjusted to the range of 0.1 to 1.0 grams.

b. The sample was aseptically transferred to a 25 x 150 mm capped sterile tube with 10 ml sterile PBS, and homogenized for 10-20 seconds with a Brinkman Polytron homogenizer equipped with a PTA 20 generator (or the equivalent).

c. Samples were placed in an ice water bath for 10-60 minutes to allow bacteria to dissociate from plant tissue.

d. After dissociation the sample was vortexed and large particles were allowed to settle, and a serial 10-fold dilution was made in sterile phosphate buffered saline.

e. Droplets (0.1 ml) of the appropriate dilutions were placed on duplicate plates of SCM.

f. Plates were incubated in the dark at 28 + 3C for 7-12 days before counting colonies.

3. Reading Plate Cultures

a. The dilution which gave counts the closest to between 8 and 80 colonies per droplet was counted.

b. A sample was considered contaminated if contaminants obscured any of the dilutions and no Cxc or Cxc/Bt was seen.

c. A sample was recorded as "0" if Cxc or Cxc/Bt was not detected and counts were not obscured by contaminants.

Calculations:

1. Inoculum Dose

Colony forming units per ml of the inoculum suspension (CFU/ml) was calculated as follows:

average count x drop factor x dilution factor = CFU/ml Where:

- average count is the average number of colonies on all plates at the dilution counted;
- drop factor is the inverse of the drop volume;
- dilution factor is the inverse of the dilution.

Needle volume was determined gravimetrically.

Inoculum dose was calculated as follows:

CFU/ml inoculum suspension x needle volume in ml = CFU inoculated.

2. Determination of CFU/g Sample

The basic formula to obtain counts in colony forming units per gram sample (CFU/g) is as follows:

(av count)(drop factor)(homogenizing volume)(dilution factor)

fresh weight in grams =CFU/g

Where:

- av count is the average number of colonies on all plates at the dilution counted;
- homogenizing volume is the volume of PBS in the homogenate;
- drop factor is the inverse of the drop volume;
- dilution factor is the inverse of dilution.

3. Log Transformation

Counts of bacteria are expressed as log10 (CFU/g). Because log10 0 is undefined, if CFU/g = 0, then log10 1 is used.

Statistical Methods:

Each plant species x plant part combination was subjected to single factor analysis for strain differences in populations of test microbes using SAS PROC GLM. Contaminated, missing, or 0 population samples were not included in the analysis. Within each of these groupings, strain means were compared using Duncan's Multiple Range at alpha = .05. Plant parts were also analyzed by SAS PROC GLM for strain differences in colonization incidence of test microbes over all plant species.

RESULTS AND DISCUSSION

Population data are presented in Table 12. Individual analysis of variance for each species x part combination showed no difference in population levels between strains in 10 cases out of 11. In the basal samples from chives, populations of the recombinant strains were significantly higher than the wild type. Distal samples from jimsonweed could not be analyzed due to the low number of colonized samples.

Incidence of colonization for each plant species x plant part x strain combination is presented in Table 13. Although the number of values is too low for a meaningful analysis of strain differences in each individual plant species x plant part combination, analysis over all species showed no significant strain differences in either the basal or the distal samples.

Careful examination of the data reveals some differences from previous experiments. Population levels and incidence of colonization in morningglory, jimsonweed, ragweed, and chive were higher than in previous experiments. Populations in distal samples of pigweed and velvetleaf were slightly lower than in previous experiments. Previous experiments had been performed in the field or in the greenhouse during

summer months. In most cases, the inoculation to harvest interval was longer than 4 weeks, and the sample size was smaller. Differences in growth conditions and inoculation to harvest interval would be expected to influence populations. Larger sample size and variation in detection limits would be expected to influence incidence of colonization.

CONCLUSION

There was very little difference in population levels and incidence of colonization between the test strain MDR1.586 and the two control strains.

Table 12

Populations of new construction MDR1.586 and control strains in basal and distal samples of various plant species measured 4 weeks after inoculation.

	Basal Samples			Distal Samples		
Plant Species	Strain	n[1]	CFU/g[2]	Strain	n[1]	CFU/g
Pigweed	MDR1.3	9	5.72 a	MDR1.3	5	4.81 a
	MDR1.586	8	5.36 a	MDE1	5	4.74 a
	MDE1	9	4.96 a	MDR1.586	7	3.76 a
Velvetleaf	MDR1.586	9	7.42 a	MDE1	8	7.37 a
	MDR1.3	9	7.38 a	MDR1.3	9	7.09 a
	MDE1	10	7.38 a	MDR1.586	7	6.80 a
Morningglory	MDE1	8	4.60 a	MDR1.3	1	3.29 a
	MDR1.3	7	4.22 a	MDE1	3	2.79 a
	MDR1.586	9	4.07 a	MDR1.586	3	2.54 a
Jimsonweed	MDR1.586	8	4.13 a	MDE1	1	3.98
	MDR1.3	6	3.57 a	MDE1.3	1	2.57
	MDE1	3	3.24 a	MDR1.586	1	2.37
Ragweed	MDR1.586	5	4.85 a	MDR1.3	1	5.31 a
	MDR1.3	7	4.33 a	MDE1.586	5	4.61 a
	MDE1	4	3.61 a	MDE1	1	4.59 a
Chive	MDR1.3	3	7.19 a	MDE1	2	3.79 a
	MDR1.586	3	6.58 a	MDR1.586	2	3.44 a
	MDE1	2	2.77	MDR1.3	0	na[3]

[1] Number of colonized samples.

[2] Mean $\log_{10}$ colony forming units per gram sample tissue of colonized samples. In each group, means followed by the same letter not significantly different by Duncan's Multiple Range Test (alpha = .05).

[3] Not applicable.

Table 13

Incidence of colonization of new construction MDR1.586 and control strains in basal and distal samples of various plant species measured 4 weeks after inoculation.

Plant Species	Strain	Basal pos/post+neg[1]	Distal pos/post+neg
Pigweed	MDE1	9/9	5/10
	MDR1.3	9/9	5/7
	MDR1.586	8/9	7/9
Velvetleaf	MDE1	10/10	8/10
	MDR1.3	9/9	9/9
	MDR1.586	9/9	7/9
Morningglory	MDE1	8/9	3/10
	MDR1.3	7/9	1/9
	MDR1.586	9/10	3/10
Jimsonweed	MDE1	3/8	1/10
	MDR1.3	6/8	1/10
	MDR1.586	8/9	1/10
Ragweed	MDE1	4/7	1/7
	MDR1.3	7/8	1/5
	MDR1.586	5/5	5/8
Chive	MDE1	2/9	2/10
	MDR1.3	3/8	0/8
	MDR1.586	3/8	2/10

[1] Number of colonized samples per number of observations.

EXAMPLE 9

DISTRIBUTION OF CXC/BT CONSTRUCT IN CORN

MATERIALS AND METHODS

Test Microbes:

The first control strain utilized in this experiment, strain MDE1, is a typical wild-type Cxc isolate. It was cultured from a lot which had been stored as 50% glycerol stocks at -20°C. This strain shows characteristic colony and cell morphology when cultured on GCab, SC and SCM, no growth on NBY, and a positive reaction to anti-Cxx antisera.

The second control strain, MDR1.3, was derived from MDE1 and contains a Bt gene, a kanamycin resistance gene, and a tetracycline resistance gene. This strain was cultured from a lot which had been stored as 50% glycerol stocks at -20°C. This strain shows characteristic colony and cell morphology when cultured on GCab, SC and SCM, as well as on these same media with tetracycline, no growth on NBY, and a positive reaction to anti-Cxx antisera.

As set forth above in Example 6, the test strain, MDR1.586, was derived from MDE1 and contains a Bt gene and a gene for tetracycline resistance. This strain was cultured from a lot which had been stored as a 50% glycerol stock at -80°C. This strain shows characteristic colony and cell morphology when cultured on GCab, SC and SCM, as well as on these same media with tetracycline, no growth on NBY, and a positive reaction to anti-Cxx antisera.

Test System:

The test system used in this experiment was a hybrid corn variety grown in 1 gallon pots in the containment greenhouse.

Inoculation:

Cells were scraped from the surface of 3-7 day old plate cultures and suspended in sterile phosphate-buffered saline at a ratio of 1 g cells: 1000 ml PBS. This suspension was inoculated into the shoot of 10 day old corn seedlings approximately 2-3 cm above the soil line. The inoculating device was the sharpened eye of a #18 or #20 hand sewing

needle held in a handle. The calculated inoculum dose was 1.7 x 107 CFU for MDE1, 9.8 x 106 CFU for MDR1.3, and 1.2 x 107 CFU for MDR1.586.

Test Method:

Inoculated plants were randomly arranged on the greenhouse bench in a split plot design with harvest dates as the main plots and strains as the subplots. There were three harvest dates, three strains, and five replications.

Sampling:

Samples were collected on dates approximately 2 weeks, 6 weeks, and 10 weeks post-inoculation. On the 2 week sampling date, plant parts assayed were: (1) the basal shoot, and (2) the highest leaf with a visible leaf collar. On the 6 and 10 week sampling dates, plant parts were: (1) the basal internode, (2) the base of the ear leaf (approximated if necessary as leaf #8 or #9), and (3) the sub-ear internode. Samples were assayed on the day of collection.

ANALYTICAL METHODS

Description of Viability Procedure:

The inoculum suspension was serially diluted in phosphate-buffered saline, and 0.1 ml droplets of the appropriate dilutions were placed on SC medium. Counts were made after 8 days incubation at 28 + 3C in the dark. The dilution which gave counts the closest to between 8 and 80 colonies per droplet was counted.

Culture Method:

1. Disinfestation

a. Samples were washed of any adhering dirt with a weak detergent solution.

b. Samples were surface disinfested by immersion (with some agitation) in a solution of 10% bleach in water with 1 drop/l Tween 80 for two minutes.

c. Samples were rinsed twice by immersion in sterile distilled water changed at appropriate intervals, and blotted dry on sterile paper towels. A minimum of about 5 mm of tissue was aseptically removed from the cut ends to

eliminate any disinfestant which may have been absorbed by the tissue.

2. Plating

a. Thin slices of the sample were aseptically weighed and adjusted to the range of 0.1 to 1.0 grams.

b. The sample was aseptically transferred to a 25 x 150 mm capped sterile tube with 10 ml sterile PBS, and homogenized for 10-20 seconds with a Brinkman Polytron homogenizer equipped with a PTA 20 generator (or the equivalent).

c. Samples were placed in an ice water bath for 10-60 minutes to allow bacteria to dissociate from plant tissue.

d. After dissociation the sample was vortexed and large particles were allowed to settle, and a serial 10-fold dilution was made in sterile phosphate buffered saline.

e. Droplets (0.1 ml) of the appropriate dilutions were placed on duplicate plates of GCab or SCM and SC + tetracycline. Samples from MDE1-inoculated plants were plated only on GCab or SCM.

f. Plates were incubated in the dark at 28 + 3C for 7-12 days before counting colonies.

3. Reading Plate Cultures

a. Counts were obtained from the GCab or SCM plates for all samples if possible. Counts were obtained from the SC + tet plates only if counts could not be made from the GCab or SCM plates.

b. The dilution which gave counts the closest to between 8 and 80 colonies per droplet was counted.

c. A sample was considered contaminated if contaminants obscured any of the dilutions and no Cxc or Cxc/Bt was seen.

d. A sample was recorded as "0" if Cxc or Cxc/Bt was not detected and counts were not obscured by contaminants.

Calculations:

1. Inoculum Dose

Colony forming units per ml of the inoculum suspension (CFU/ml) was calculated as follows:

average count x drop factor x dilution factor = CFU/ml

Where:

- average count is the average number of colonies on all plates at the dilution counted
- drop factor is the inverse of the drop volume
- dilution factor is the inverse of the dilution.

Needle volume was determined gravimetrically and rounded to the nearest ul.

Inoculum dose was calculated as follows:

CFU/ml inoculum suspension x needle volume in ml = CFU inoculated.

2. Determination of CFU/g Sample

The basic formula to obtain counts in colony forming units per gram sample (CFU/g) is as follows:

(av count)(drop factor)(homogenizing volume)(dilution factor)

fresh weight in grams =CFU/g

Where:

- av count is the average number of colonies on all plates at the dilution counted;
- homogenizing volume is the volume of PBS in the homogenate;
- drop factor is the inverse of the drop volume;
- dilution factor is the inverse of dilution.

3. Log Transformation

Counts of bacteria are expressed as log10 (CFU/g). Because log10 0 is undefined, if CFU/g = 0, then log10 1 is used.

Statistical Methods:

Each harvest date x plant part combination was subjected to single factor analysis for strain differences using SAS PROC GLM. Contaminated, missing, or 0 population samples

were not included in the analysis. Within each of these groupings, strain means were compared using Duncan's Multiple Range at alpha = .05.

RESULTS AND DISCUSSION

Log10-transformed population data for each of the strains for each sampling date x plant part combination are compared in Table 14. Basal shoot and basal internode population dynamics are graphically presented in Figure 18.

Strain populations were compared in 8 sampling date x plant part combinations. There was relatively little difference between strains in any of the comparisons. In 5 instances out of 8, there were no significant differences in populations between any of the strains. The only evident trend was that in 7 of the 8 comparisons, populations of the wild-type control (MDE1) were lower than all other strains. Only in 3 of these instances, however, were these populations significantly different from any of the other strains.

CONCLUSIONS

There was relatively little variation between populations of the three strains tested in this experiment.

Table 14

Populations of new construction MDR1.586 and control strains in various plant parts of greenhouse grown corn, ranked in descending order.

a. 2 Weeks Post-Inoculation

Basal Shoot		Highest Leaf	
Strain	CFU/g[1]	Strain	CFU/g
MDR1.586	7.50 a	MDR1.586	7.00 a
MDE1	7.12 a	MDE1	6.82 a
MDR1.3	nd[2]	MDR1.3	nd
c.v.[3] = 8.1%		c.v. = 5.4%	

b. 6 Weeks Post-Inoculation

Basal Internode		Sub-ear Internode		Ear Leaf	
Strain	CFU/g[1]	Strain	CFU/g[1]	Strain	CFU/g[1]
MDR1.3	8.57 a	MDR1.3	8.40 a	MDR1.3	7.13 a
MDR1.586	8.15 ab	MDR1.586	8.08 ab	MDR1.586	7.11 a
MDE1	8.00 b	MDE1	7.80 b	MDE1	6.63 a
c.v. = 4.2%		c.v. = 4.9%		c.v. = 7.2%	

c. 10 Weeks Post-Inoculation

Basal Internode		Sub-ear Internode		Ear Leaf	
Strain	CFU/g[1]	Strain	CFU/g[1]	Strain	CFU/g[1]
MDR1.3	9.02 a	MDR1.586	9.21 a	MDR1.3	9.57 a
MDR1.586	8.99 a	MDR1.3	9.06 a	MDR1.586	9.53 a
MDE1	8.82 a	MDE1	9.04 a	MDE1	8.99 b
c.v. = 2.4%		c.v. = 4.2%		c.v. = 4.0%	

[1] Mean $\log_{10}$ CFU/g. Means followed by same letter are not significantly different by Duncan's Multiple Range Test (alpha = .05).

[2] Not done.

[3] Coefficient of variation.

EXAMPLE 10

THE PRODUCTION OF BT ANTIGEN BY A CXC/BT CONSTRUCT

This study assessed the amount and molecular weight distribution of Bt endotoxin made by a new Cxc/Bt construction. The test microbe consists of a Maryland strain of Clavibacter xyli subsp. cynodontis (Cxc) that has been genetically engineered to produce the delta endotoxin of Bacillus thuringiensis subsp. kurstaki ("Bt") and designated as MDR1.586.

MATERIALS AND METHODS

Test Microbe

The test microbe used in this study, MDR1.586, is described above in Example 6.

Test System

The basis for quantitation of total Bt antigen in strain MDR1.586 was immunovisualization of Bt protoxin on Western Blots (1) of SDS-Polyacrylamide gels. This technique allowed the determination of a standard curve relating amounts of pure solubilized Bt crystal protein with the intensities of color produced with an enzyme-linked second antibody. Quantitative densitometry was employed to determine values of color intensity. The test cell extract samples were solubilized and treated identically to the Bt standards, and the amount of Bt antigen determined by reference to the standard curve.

Bt antigen produced by Cxc reflects degradation products of the Bt molecule as well as the expected 130 kd protein coded by the native Bt gene. The distribution of molecular weight species in strain MDR1.586 was also determined by scanning densitometry.

ANALYTICAL METHODS

Preparation of Cxc Cell Extracts

A culture of strain MDR1.586 was grown in 50 ml S27 medium to mid-log (A660 = 0.5) at 30°C in shaken, baffled flasks. The culture was chilled on ice, and cells were harvested by centrifugation. The cells were washed with a solution containing TRIS-HCl (25mM, pH 6.8), leupeptin (0.5

sg/ml), phenylmethyl sulfonyl fluoride (34 sg/ml) and disodium EDTA (1 mM) at 0°C. They were then resuspended in 1 ml of the same solution. The cells were disrupted by 3 min ballistic disintegration in a "Bead-Beater" apparatus (BioSpec Products, Bartlesville, OK). The resulting lysates were centrifuged lightly to sediment the glass beads, and the extracts stored at -20°C.

Quantitation of Protein

Crude cell protein and Bt crystal protein were determined by the Bradford method (Bio-Rad) with bovine serum albumin as reference. Both cell extract samples and Bt crystals were solubilized with 5% NH4OH and 12.5 mM mercaptoethanol prior to assay.

Preparation of the Bt Crystal Protein Standard

Bt crystals were prepared from B. thuringiensis subsp. kurstaki HD73 and purified on Renograffin gradients.

Immunochemical Detection of Bt antigen

For quantitation and molecular weight distribution analysis of Bt antigen, samples of the cell extracts were solubilized in 1% SDS sample buffer and applied to 4-20% gradient polyacrylamide-SDS gels (Diichi Pure Chemicals, Tokyo, Japan). After electrophoresis the gels were Western blotted (1) onto Immobilon paper (Millipore, Inc.) in an LKB Transblot apparatus according to manufacturer's instructions. The Immobilon blot was blocked with skim milk 18 hr at 21°C. The first antibody, a goat anti-Bt preparation, was added to the paper and incubated 3 hr at 21°C. After 4 washes to remove the first antibody a second antibody, rabbit anti-goat IgG conjugated with alkaline phosphatase (KPL laboratories), was added and incubated 1 hr at 21°C. The second antibody was removed by washing and the color reaction initiated by the addition of the colorigenic substrates (KPL laboratories). After development the filter was washed with water to halt the reaction. The filters were stored dry prior to scanning densitometry.

Densitometry

Filters with immunochemically visualized Bt antigen were scanned with an LKB Model 2222-020 Ultroscan. The density output in area (absorbence units x mm2) was used to construct the Bt standard curve and to compute values for the cell extract samples including the relative contribution of each peak to the total antigenic profile.

RESULTS

Quantitation of total Bt antigen produced by strain MDR1.586.

Fig. 19 shows the standard curve of densitometric units as a function of 130 kdalton Bt crystal protein. Table 15 shows the absorbence values obtained for the 130 kdalton Bt protoxin of the cell extracts, and the conversion of these to weights of Bt protoxin. The corresponding sample values for MDR1.586 cell extracts give a value of 1.3% as the amount of Bt protoxin in cell extract protein.

Contribution of different molecular weight forms of Bt antigen to the total expression.

Fig. 20 shows the scan of Bt antigen as a function of mobility of the different bands. Quantitation of peak areas gave the relative proportions of each band to the total. The major band (32% of total antigen) is the 130 kd native Bt protoxin.

CONCLUSIONS

Strain MDR1.586 produces approximately 10-fold more Bt than the previously found with strain MDR1.3 and this is reflected in the increased toxicity to the European Corn Borer in planta (see Example 7).

Table 15

Quantitation of 130 Kdalton Bt protoxin in extracts of strain MDR1.586

Extract Sample	Sample Protein (ng)	Area (130 kd Peak)	Protoxin[a] (ng protein)	Percent Total Protein
1	7690	0.37	107	1.3
2	7690	0.36	107	1.3

[a] Computed from standard curve, Figure 1.

While the processes and products of the present invention have been described primarily in reference to hybrids of bacteria capable of fixing nitrogen or producing avermectins, it is to be understood that, with appropriate changes in the screening procedure, hybrids capable of producing other agricultural chemicals, such as insecticides, herbicides, antifungal agents, antibacterial agents, antiviral agents, plant growth regulators, and solubilized phosphates can be prepared in accordance with the present invention. Moreover, while the foregoing examples illustrate the inclusion of genes for production of one agricultural chemical in the hybrid, it is envisioned that genes for production of two or more compatible products can be included in hybrids found in accordance with the present invention. Thus, fusion of hybrids formed in accordance with the present invention with other agricultural-chemical-producing microorganisms, initial fusion with microorganisms known to produce more than one agricultural chemical, or initial fusion conducted with more than one agricultural chemical producing microorganism would yield such multi-chemical-producing organisms. Similarly, two or more genes for the production of agricultural chemicals can be cloned into an infecting microorganism, using recombinant DNA techniques. For example, the genes for the delta-endotoxins of Bacillus, thuringiensis var. kurstaki and Bacillus thuringiensis var. tenebrionis could be cloned into an infecting microorganism.

In addition, the scope of applicable plant hosts can be expanded by combining the genetic material of two or more infecting microorganisms.

It will be apparent to those skilled in the art that various modifications and variations can be made in the processes and products of the present invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided that they come within the scope of the appended claims and their equivalents.

WHAT IS CLAIMED IS:

1. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host comprising:

(A) in any convenient order:
  (1) identifying said plant host; and
  (2) identifying an infecting microorganism which infects said plant host;

(B) preparing a vector capable of being transferred into and replicating in said infecting microorganism;

(C) preparing an expression module capable of directing the production of an agricultural chemical by said infecting microorganism;

(D) placing said expression module in said vector to create an expression vector capable of being transferred into and replicating in said infecting microorganism, said expression vector being capable of directing the production of said agricultural chemical by said infecting microorganism;

(E) transforming said infecting microorganism with said expression vector to produce hybrid microorganisms;

(F) selecting for said hybrid microorganisms;

(G) selecting or screening serially from said selected hybrid microorganisms and in any convenient order:
  (1) a subgroup comprising those hybrid microorganisms which manifest the ability to interact with plant tissue in the manner in which said infecting microorganism interacts with plant tissue during the initial phase of infection in said plant host;
  (2) a subgroup comprising those hybrid microorganisms which, upon application to

said host, do not create manifestations of disease; and

(3) a subgroup comprising those hybrid microorganisms having the ability to produce said agricultural chemical if not previously selected for; and

(H) selecting hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host by selecting from the products of the last performed step of steps (G)(1) to (G)(3) those hybrid microorganisms capable of improving the performance of said plant host under conditions wherein said performance would be improved by direct application of said agricultural chemical to said plant host;

wherein said expression module comprises a gene obtained from the group consisting of microorganisms, plants, vertebrates, and invertebrates.

2. The method of claim 1 wherein said step of preparing an expression module comprises:

preparing a portable DNA sequence containing the structural gene or genes for said agricultural chemical;

cloning said portable DNA sequence into a vector containing transcription and translation control elements for said portable DNA sequence, said control elements being operable in said infecting microorganism, to create an expression module, said expression module being capable of directing said infecting microorganism to produce said agricultural chemical; and

recovering said expression module.

3. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host comprising:

(A) in any convenient order:

(1) identifying said plant host; and

(2) identifying an infecting microorganism which infects said plant host;

(B) preparing an integration vector containing an integration sequence and capable of integrating into the genome of said infecting microorganism;

(C) preparing an expression module capable of directing the production of an agricultural chemical by said infecting microorganism;

(D) placing said expression module within aid integration sequence of said integration vector, thereby producing a modified integration vector capable of integrating into the genome of said infecting microorganism and directing the production of said agricultural chemical by said infecting microorganism;

(E) transforming said infecting microorganism with said modified integration vector to produce hybrid microorganisms;

(F) selecting for said hybrid microorganisms;

(G) selecting serially from said selected hybrid microorganisms and in any convenient order:

(1) a subgroup comprising those hybrid microorganisms which manifest the ability to interact with plant tissue in the manner in which said infecting microorganism interacts with plant tissue during the initial phase of infection in said plant host;

(2) a subgroup comprising those hybrid microorganisms which, upon application to said host, do not create manifestations of disease; and

(3) a subgroup comprising those hybrid microorganisms having the ability to produce said agricultural chemical if not previously selected for; and

(H) selecting hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host by selecting from the products of the last performed step of steps (G)(1) to (G)(3) those hybrid microorganisms capable of improving the performance of said plant host under conditions wherein said performance would be improved by direct application of said agricultural chemical to said plant host;

wherein said expression module comprises a gene obtained from the group consisting of microorganisms, plants, vertebrates, and invertebrates.

4. The method of 3 wherein said expression module comprises a portable DNA sequence containing the structural gene or genes for the production of said agricultural chemical and transcription and translation control elements, said control elements being operable in said infecting microorganism.

5. The method of 4 wherein said expression module further comprises a DNA sequence that codes for a selectable trait in said infecting microorganism.

6. The method of 3 wherein said step of preparing an expression module comprises:

preparing a vector containing a promoter that is operable in said infecting microorganism;

placing into said vector a portable DNA sequence containing the structural gene or genes for the production of said agricultural chemical and containing transcription and translation control elements other than a promoter said control elements being operable in said infecting microorganism, to produce an expression vector containing an expression module, said expression module comprising said portable DNA sequence and said control elements and said expression module being operable in said infecting microorganism; and

recovering said expression module from said expression vector.

7. The method of claim 6 comprising the further step of placing into said vector a DNA sequence that codes for a selectable trait in said infecting microorganism, wherein said expression module further comprises said DNA sequence that codes for said selectable trait.

8. The method of claim 7 wherein said DNA sequence that codes for a selectable trait in said infecting microorganism is within said expression module.

9. The method of claim 3 wherein said infecting microorganism is a microorganism selected from the group consisting of bacteria, fungi, and alga.

10. The method of claim 9 wherein the bacteria is of a genus selected from the group consisting of Acetobacter, Agrobacteria, Azospirillum, Clavibacter, Corynebacteria, Erwinia, Herbispirillum, Pseudomonas, Streptomyces, Xanthomonas, and xylem-limited bacteria.

11. The method of claim 10 wherein said Clavibacter is of the species xyli.

12. The method of claim 11 wherein said plant host is of the Gramineae family.

13. The method of claim 12 wherein said plant host is bermuda grass, sugar cane, or sorghum.

14. The method of claim 11 wherein said plant host is corn.

15. The method of claim 11 wherein said bacterium is Clavibacter xyli subsp. cynodontis and said plant host is corn.

16. The method of claim 11 wherein said bacterium is Clavibacter xyli subsp. cynodontis and said plant host is corn.

17. The method of claim 3 wherein said agricultural chemical is a crop protection agent selected from the group consisting of lysozymes, 1,3-glucanases, chitinases, cecropins, attacins, magainins, protease inhibitors, and polyketides.

18. The method of claim 3 wherein said integration vector is pCG300.

19. The method of claim 6 wherein said vector containing a promoter is pCG6.

20. A stable hybrid microorganism having the ability to produce an agricultural chemical and the ability to enter into nonpathogenic, endosymbiotic relationships with a plant host, wherein said agricultural chemical is a crop protection agent selected from the group consisting of lysozymes, 1,3-glucanases, chitinases, cecropins, attacins, magainins, protease inhibitors, and polyketides.

21. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 1.

22. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 3.

23. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 11.

24. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 12.

25. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 13.

26. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 14.

27. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 15.

28. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 16.

29. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 17.

30. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 18.

31. The hybrid agricultural-chemical-producing-microorganism produced by the method of claim 19.

32. An agricultural seed product comprising crop plant seed, a biodegradable nutrient carrier material coated on said seed, and a stable hybrid microorganism having the ability to produce agricultural chemicals and to enter into

nonpathogenic endosymbiotic relationships with said crop plant associated with said carrier material wherein said agricultural chemical is a crop protection agent selected from the group consisting of lysozymes, 1,3-glucanases, chitinases, cecropins, attacins, magainins, protease inhibitors, and polyketides.

33. An agricultural product comprising crop plant seed infected with a stable hybrid microorganism having the ability to produce agricultural chemicals and to enter into nonpathogenic endosymbiotic relationships with said crop plant, wherein said agricultural chemical is a crop protection agent selected from the group consisting of lysozymes, 1,3-glucanases, chitinases, cecropins, attacins, magainins, protease inhibitors, and polyketides.

34. An agricultural soil drench comprising a mixture of water and a stable hybrid microorganism having the ability to produce agricultural chemicals and to enter into nonpathogenic, endosymbiotic relationships with a plant host, wherein said agricultural chemical is a crop protection agent selected from the group consisting of lysozymes, 1,3-glucanases, chitinases, cecropins, attacins, magainins, protease inhibitors, and polyketides.

35. An agricultural product comprising a stable hybrid microorganism having the ability to produce agricultural chemicals and the ability to enter into nonpathogenic, endosymbiotic relationships with a plant host in a horticulturally acceptable carrier suitable for injection into said plant host, wherein said agricultural chemical is a crop protection agent selected from the group consisting of lysozymes, 1,3-glucanases, chitinases, cecropins, attacins, magainins, protease inhibitors, and polyketides.

36. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 5, 7, or 8, wherein said selectable trait of the infecting microorganism is selected from a group consisting of antibiotic resistance, need for nutritional

supplementation, resistance to toxins, or combinations thereof.

37. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein the ability to interact with plant tissue in the manner in which said infecting microorganism interacts with plant tissue during the initial phase of infection in said plant host is an ability selected from the group consisting of the ability to bind to plant cells, the ability to spread through the vascular system of said plant host, and the ability to live in the roots of the plant host without creating symptoms of disease.

38. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a monocotyledonous plant.

39. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a temperate cereal crop plant selected from the group consisting of wheat, triticale, barley, rye or oats.

40. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a grass selected from the group consisting of brome grass, bluegrass, tall fescue grass and bermuda grass.

41. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a tropical grass selected from the group consisting of sugar cane, corn, millet or sorghum.

42. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is rice.

43. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a dicotyledonous plant.

44. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 43, wherein said plant host is a solanaceous plant selected from the group consisting of potatoes, tomatoes, tobacco, eggplant or pepper.

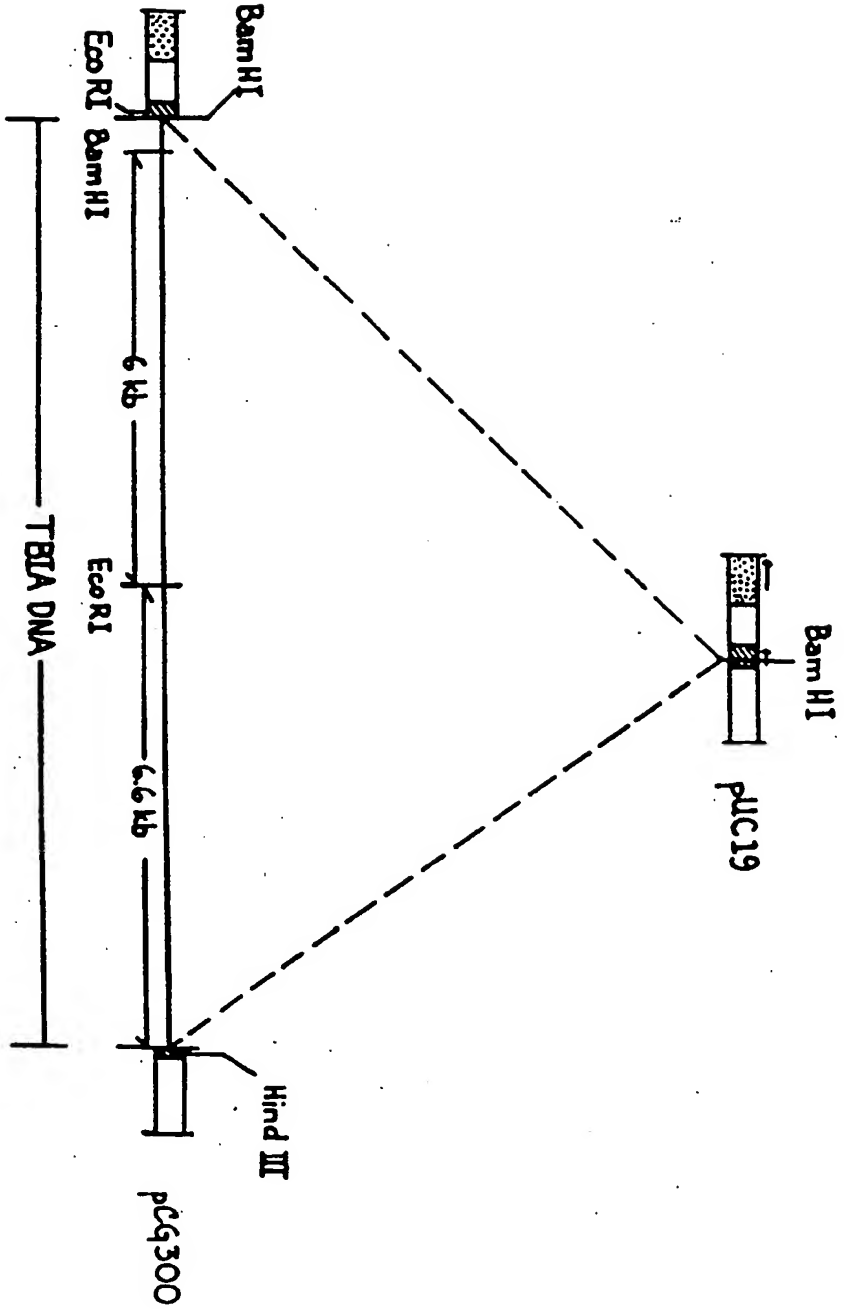
45. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 43, wherein said plant host is a brassicaceous plant selected from the group consisting of cauliflower, broccoli, cabbage, kale, or kohlrabi.

46. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 43, wherein said plant host is a vegetable selected from the group consisting of carrot or parsley.

47. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 43, wherein said plant host is an agriculturally grown plant selected from the group consisting of sugar beets, cotton, fruit trees, berry plants or grapes.

48. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 43, wherein said plant host is a tree species selected from the group consisting of pine, spruce, fir or aspen.

49. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a legume selected from the group consisting of soybeans, alfalfa, clover, field beans, mung beans, peas and other pulses.


Bam HI
Eco RI Bam HI
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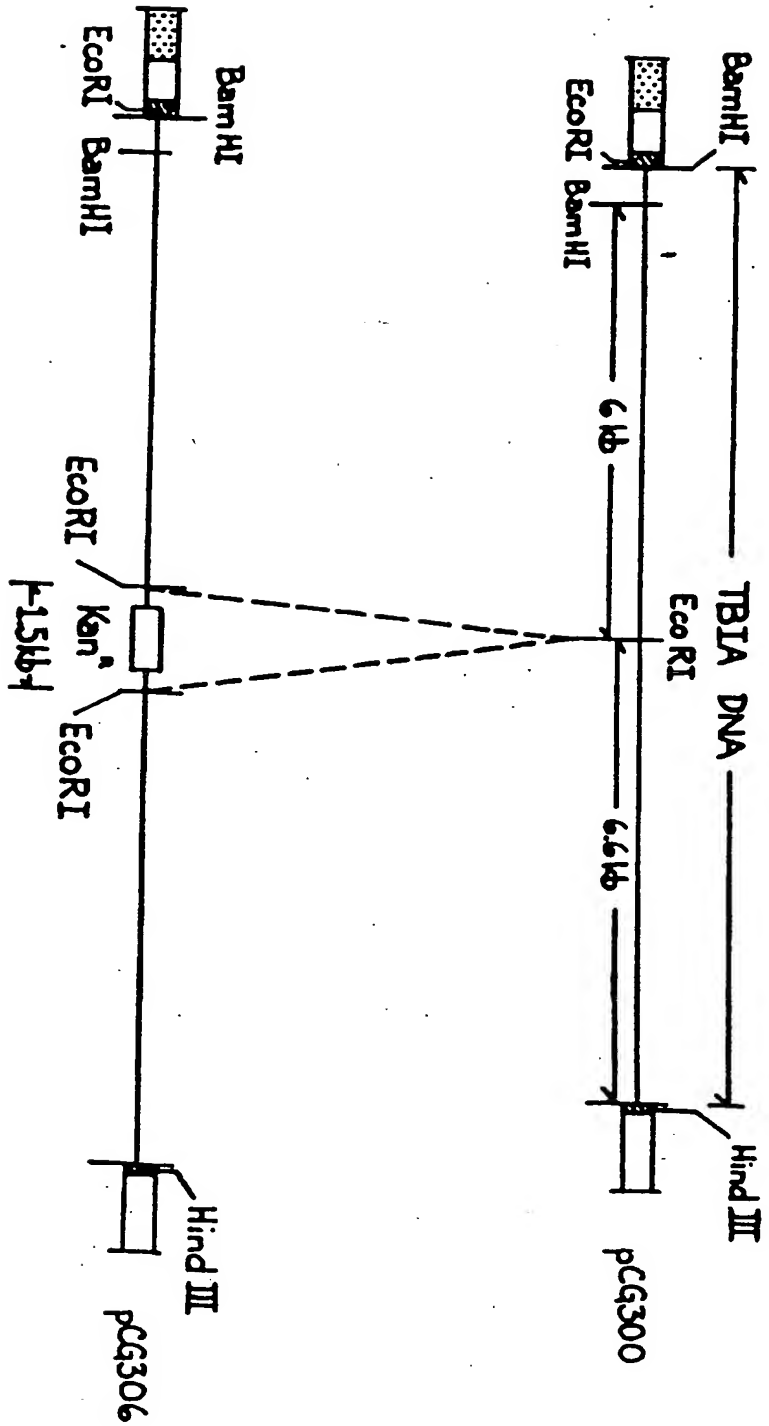
Eco RI
6.6 kb
Hind III
TBIA DNA
pCG300
Bam HI
pUC19
= LacZ
= Ap^R

(Figure 1)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EcoRI BamHI
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TBIA DNA
EcoRI
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Hind III
pCG300
BamHI
EcoRI BamHI
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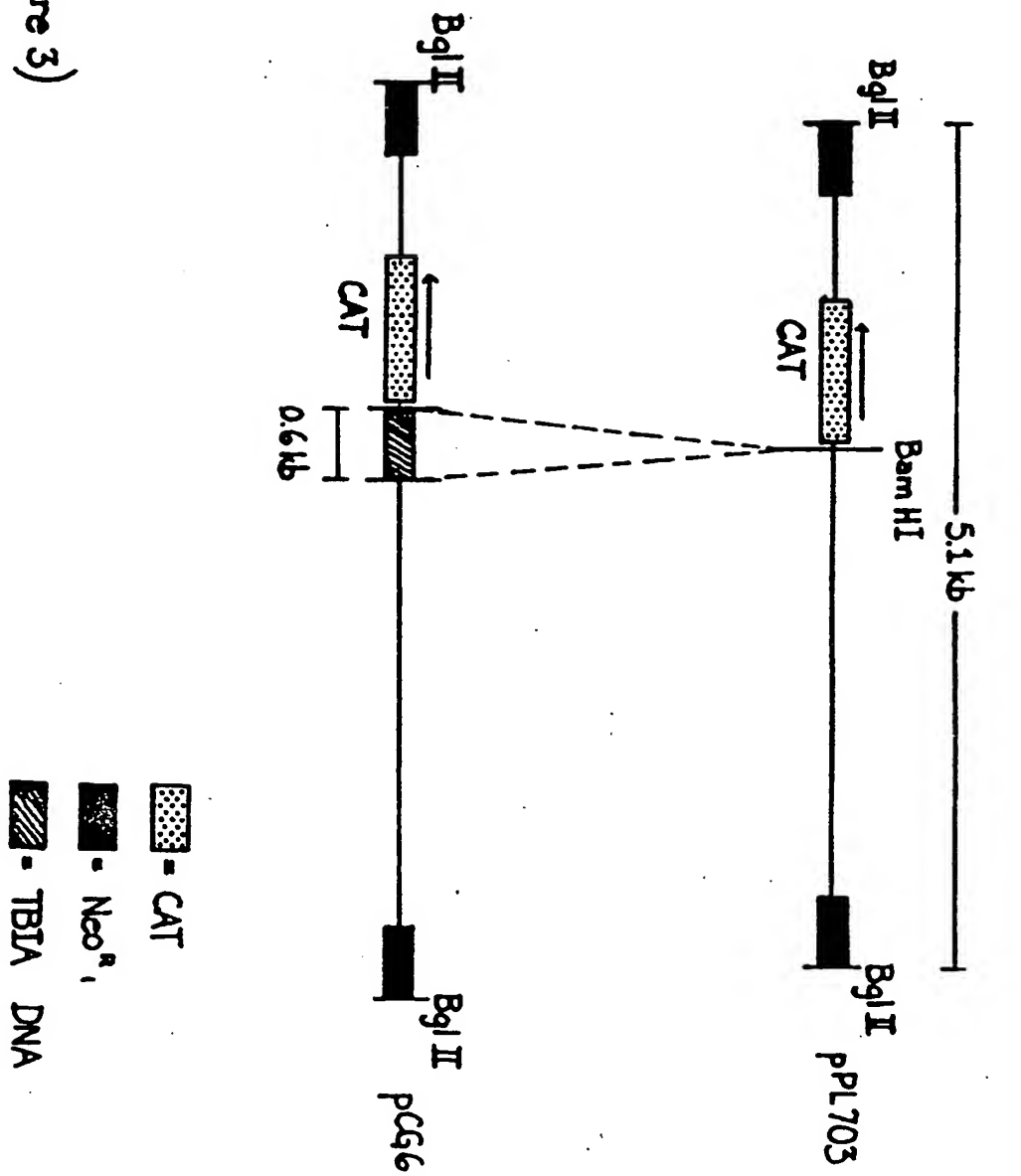
= Ap^R

(Figure 2)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CAT
0.6 kb
Bgl II
PCG6
Bgl II
CAT
Bam HI
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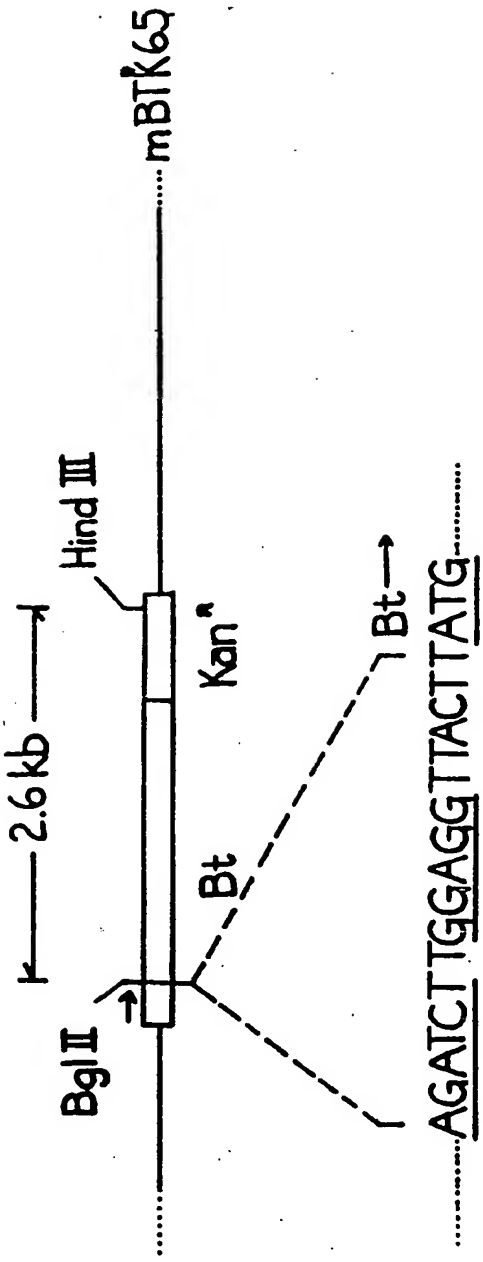
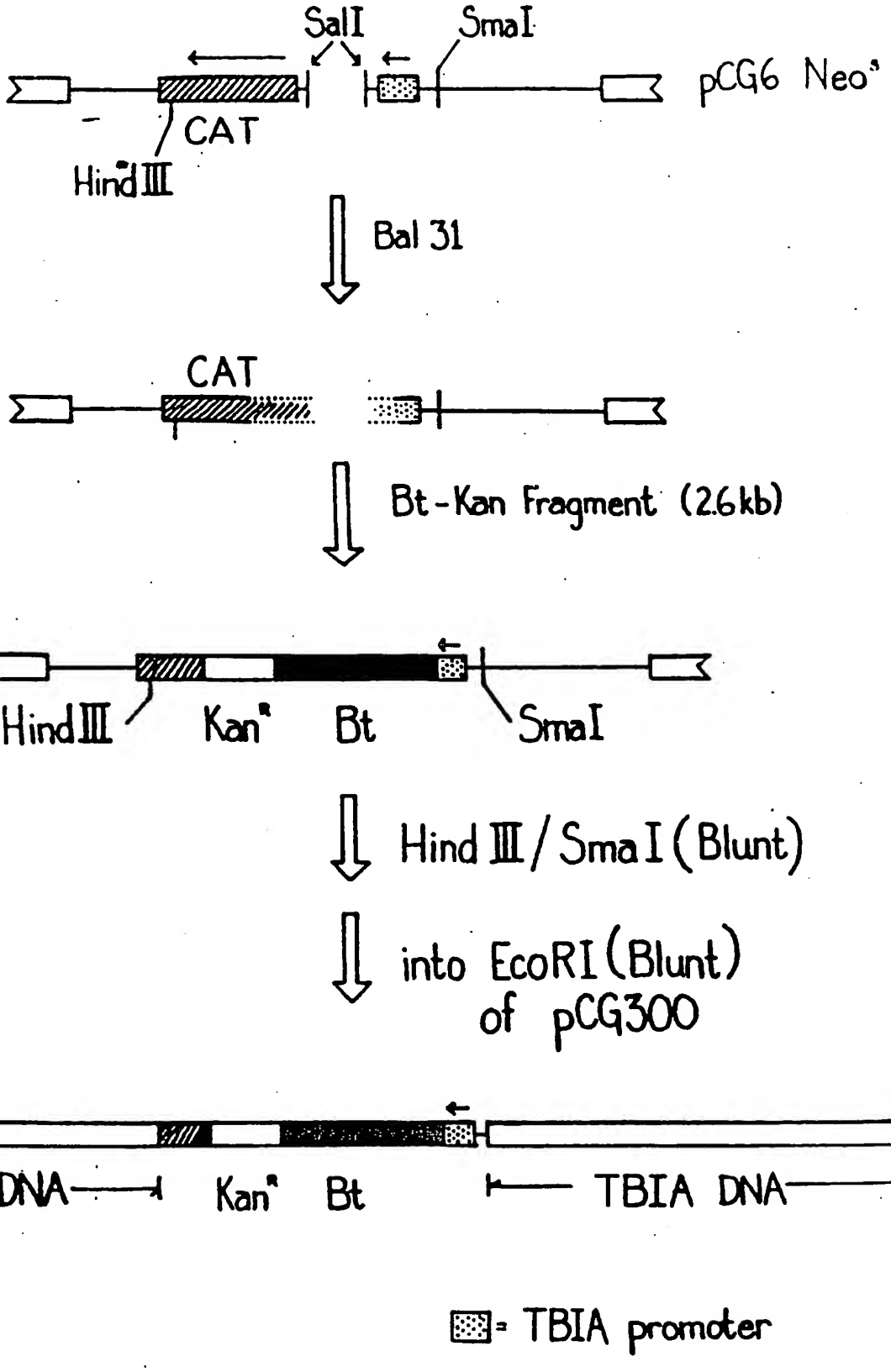
Bgl II
PPL703
= CAT
= Neo^R
= TBIA DNA

(Figure 3)


mBTK65
Hind III
2.6 kb
Kan^R
Bt
Bt
Bgl II
AGATCTTGGAGGTTACTTATG
Bgl II
S-D
Met

(Figure 4)

(Figure 5)


SalI
SmaI
pCG6 Neo^s
CAT
Hind III
Bal 31
CAT
Bt-Kan Fragment (2.6kb)
Hind III
Kan^R
Bt
SmaI
Hind III / Sma I (Blunt)
into EcoRI (Blunt)
of pCG300
TBIA DNA
Kan^R
Bt
TBIA DNA
= TBIA promoter

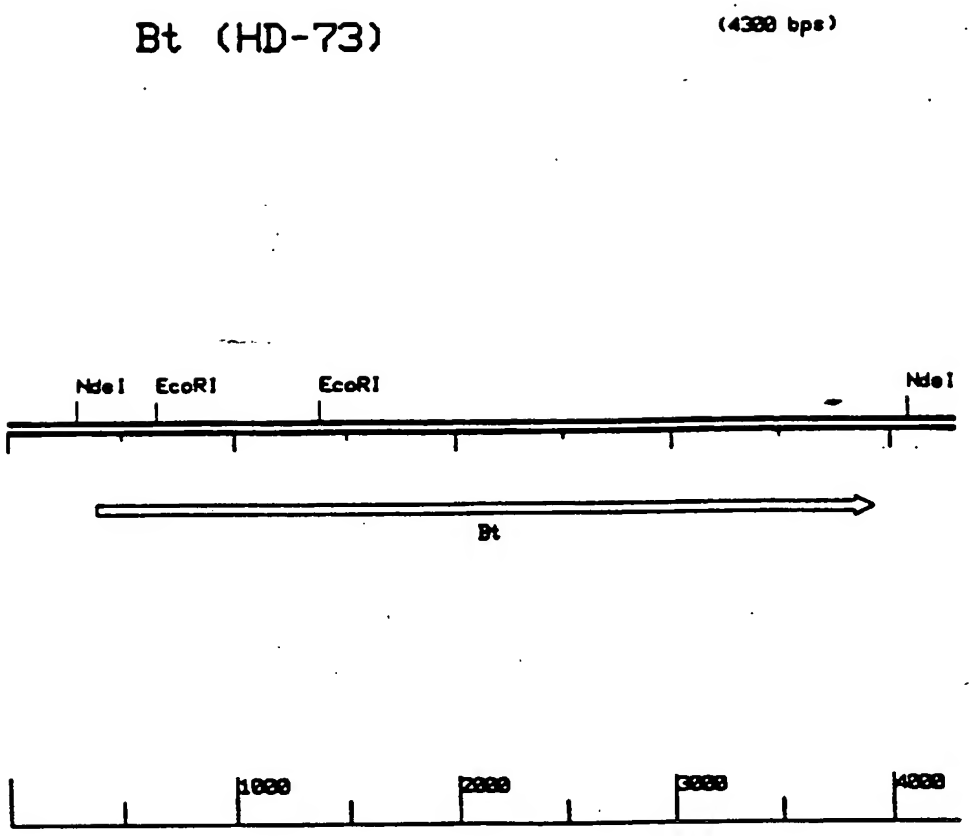

Bt (HD-73)
(4300 bps)
NdeI
EcoRI
EcoRI
NdeI
Bt
1000
2000
3000
4000
Base Pairs

Figure 6

Sequence of 4.993 HincII Fragment of Tn916.

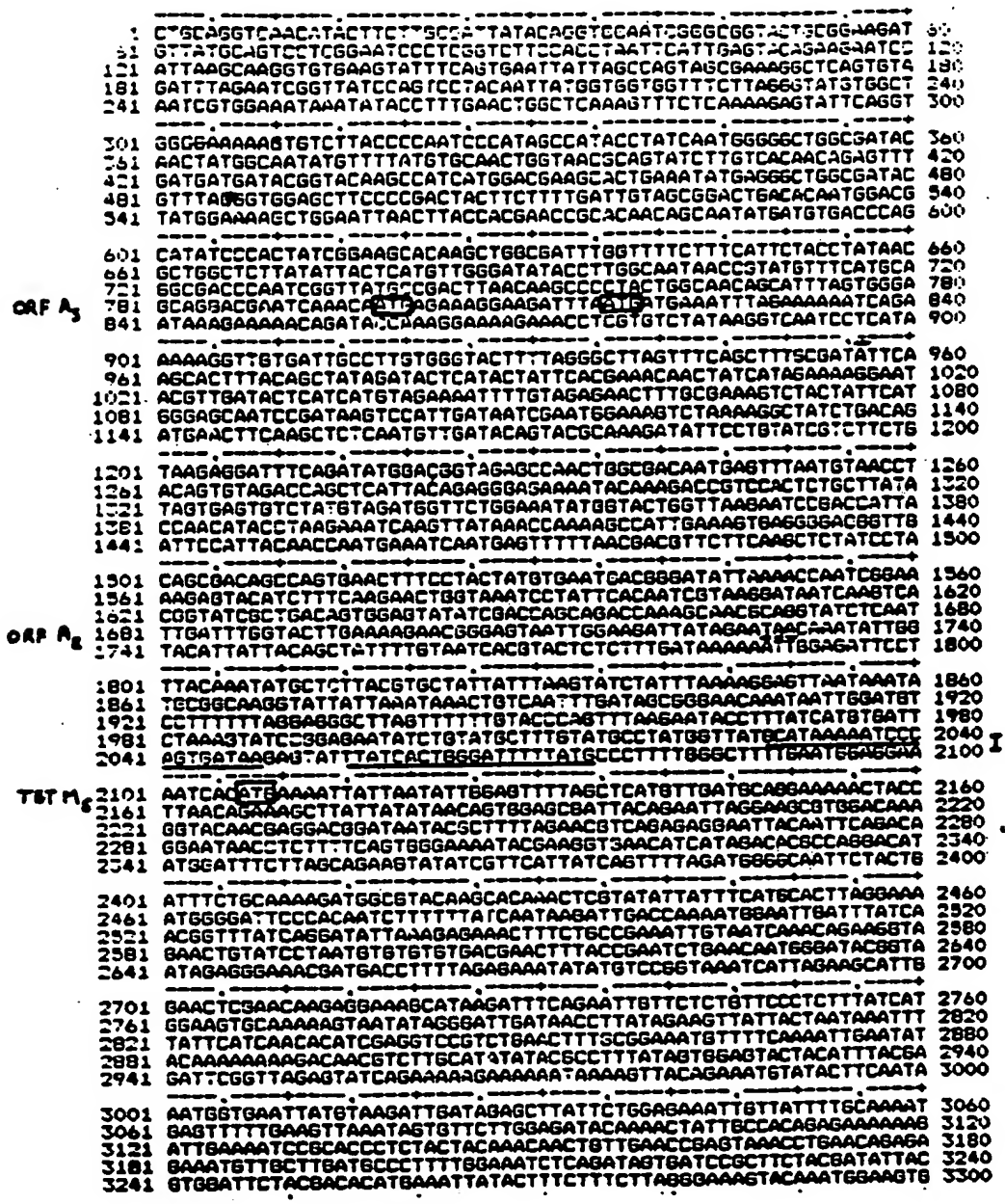


Figure 7

ORF A_s = ATG start of putative gene A (box).
ORF A_e = Termination codon of putative gene A (dashes).
ORF B_s = ATG start of putative gene B (complement) (box).
ORF B_e = Termination codon of putative gene B (complement) (dashes).
IR = Inverted repeat (underline).
$TetM_s$ = ATG start of tetM gene (box).
$TetM_e$ = Termination codon of tetM gene (dashes).

Figure 7
(continued)

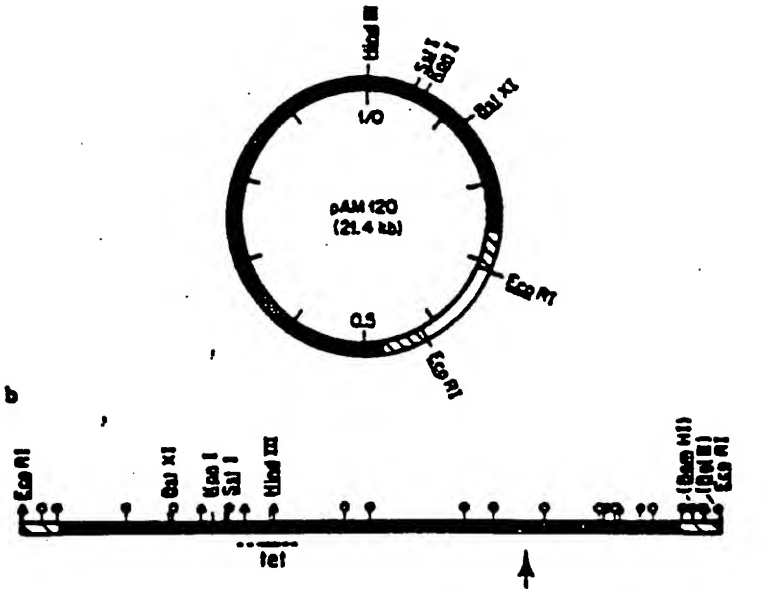


Figure 1 Restriction map of Tn*916*. (*a*) pAM120, representing the *Eco*RI fragment F::Tn*916* from pAD1::Tn*916* (pAM211) cloned into the *E. coli* vector pGL101. The dark segment represents Tn*916*. The striped segments represent the portions of the *Eco*RI F fragment flanking Tn*916*. The remainder represents pGL101. (*b*) Tn*916* and its flanking DNA. The restriction sites are indicated or are represented by: filled circle, *Sau*3A; open circle, *Hin*cII; triangle, *Hpa*II. The *Hin*dIII site in Tn*916* is believed to be within or near *tet*.

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Figure 8

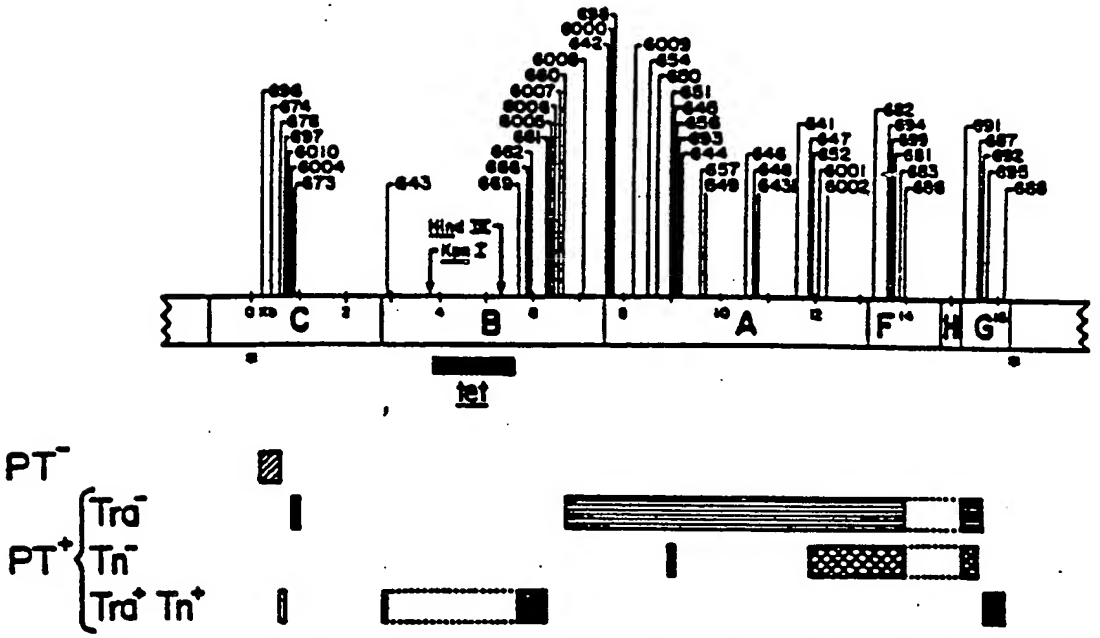

FIG. L. Tn*916* restriction map showing insertions of Tn*5* and resulting effects on behavior. The asterisks mark the ends of the transposon. The segments marked A (5.5 kb), B (4.3 kb), C (3.6 kb), F (1.6 kb), G (1.1 kb), and H (0.4 kb) correspond to *Hin*cII fragments originally identified in pAM120 (14, 22). The numbers correspond to the corresponding pAM derivatives (Table 3) generated. The different behaviors are indicated in Table 3 as follows: PT⁻, unable to transform *S. faecalis* OG1X protoplasts; Tra⁻, unable to transfer conjugatively; Tn⁻, unable to detect intracellular transposition; and PT⁺ Tra⁺ Tn⁺, behavior similar to wild-type Tn*916*. The resistance determinant is indicated as *tet*.

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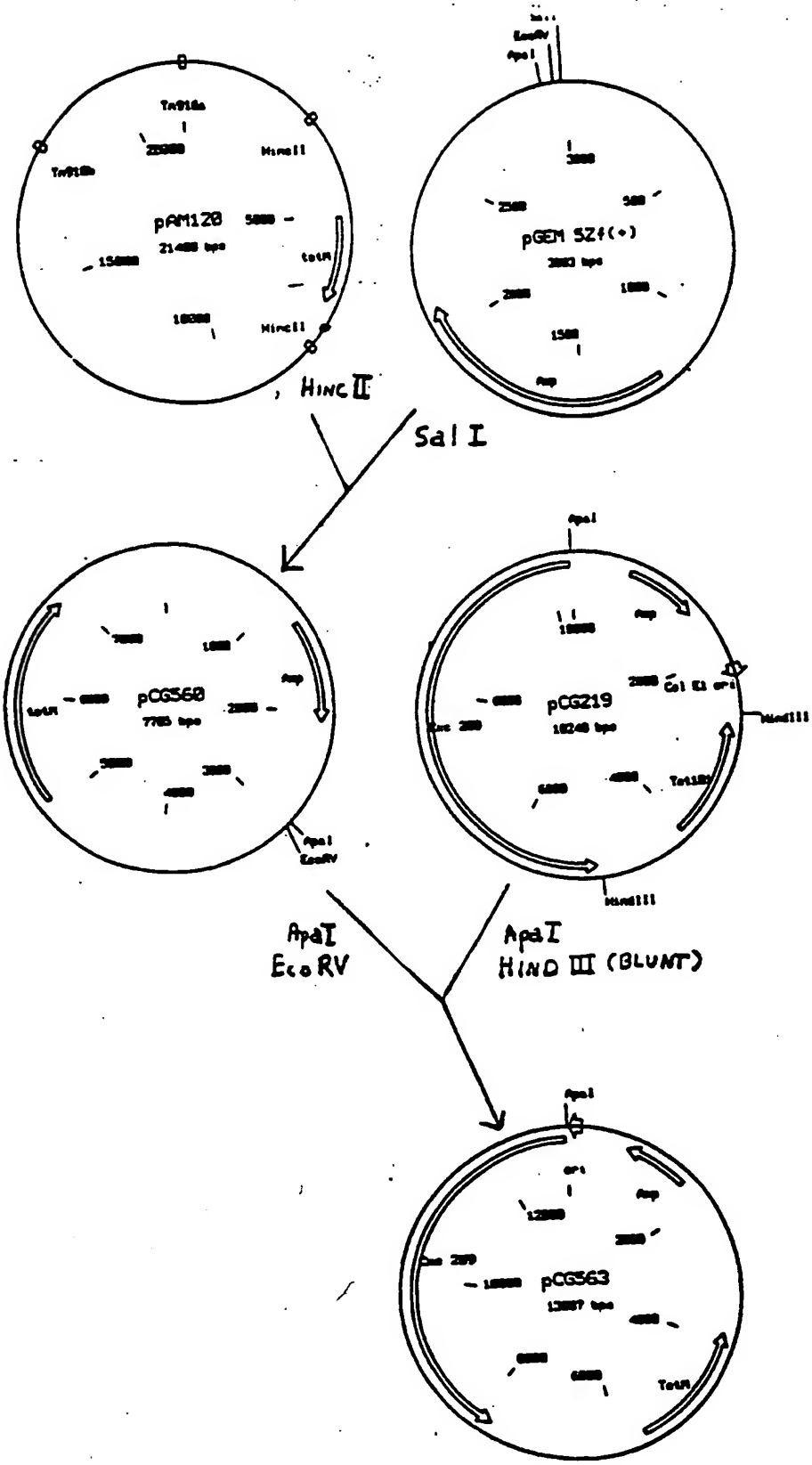
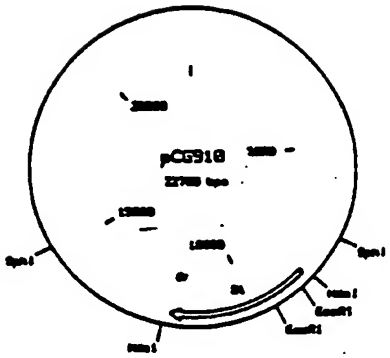
Figure 9



Assembly of pCG563.

Figure 10



Modification of Bt gene.

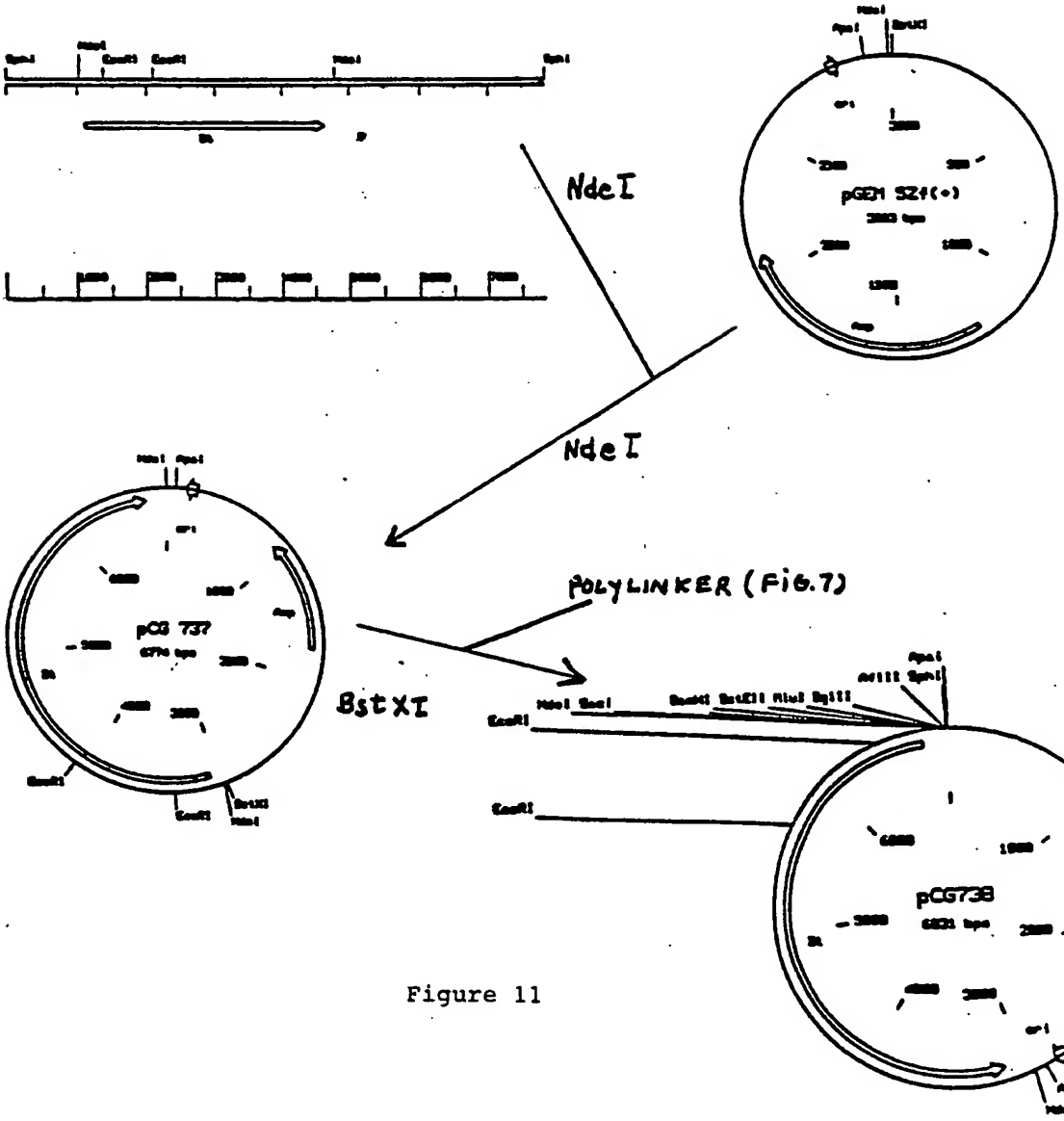
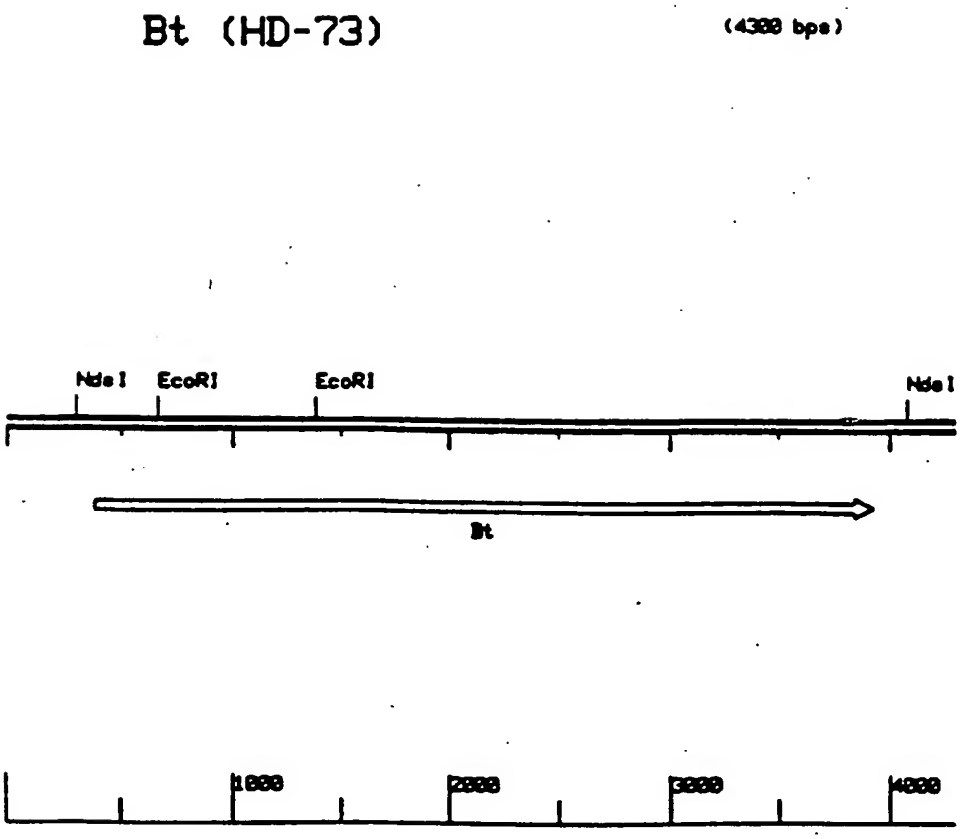


Figure 11



Comparison of sequence 5' to amino terminus of native and modified Bt genes.

5' ApaI AflII MluI BamHI
GGGCCCGCATGCCTTAAGATCTACGCGTGGTAACCGGATCCTAGCTAATTTGACGCGTTGG....Modified
SphI BglII BstEII

SacI
GAGCTCTCCCATATGTTTTAAATTGTAGTAATGAAAAACAGTATTATATCATAATGAATT...Modified
NdeI
.....CATATGTTTTAAATTGTAGTAATGAAAAACAGTATTATATCATAATGAATT...Native

GGTATCTTAATAAAAGAGATGGAGGTAACTTATGGAT.....Modified

GGTATCTTAATAAAAGAGATGGAGGTAACTTATGGAT.....Native
Bt

Figure 12

Final Assembly . pCG741.

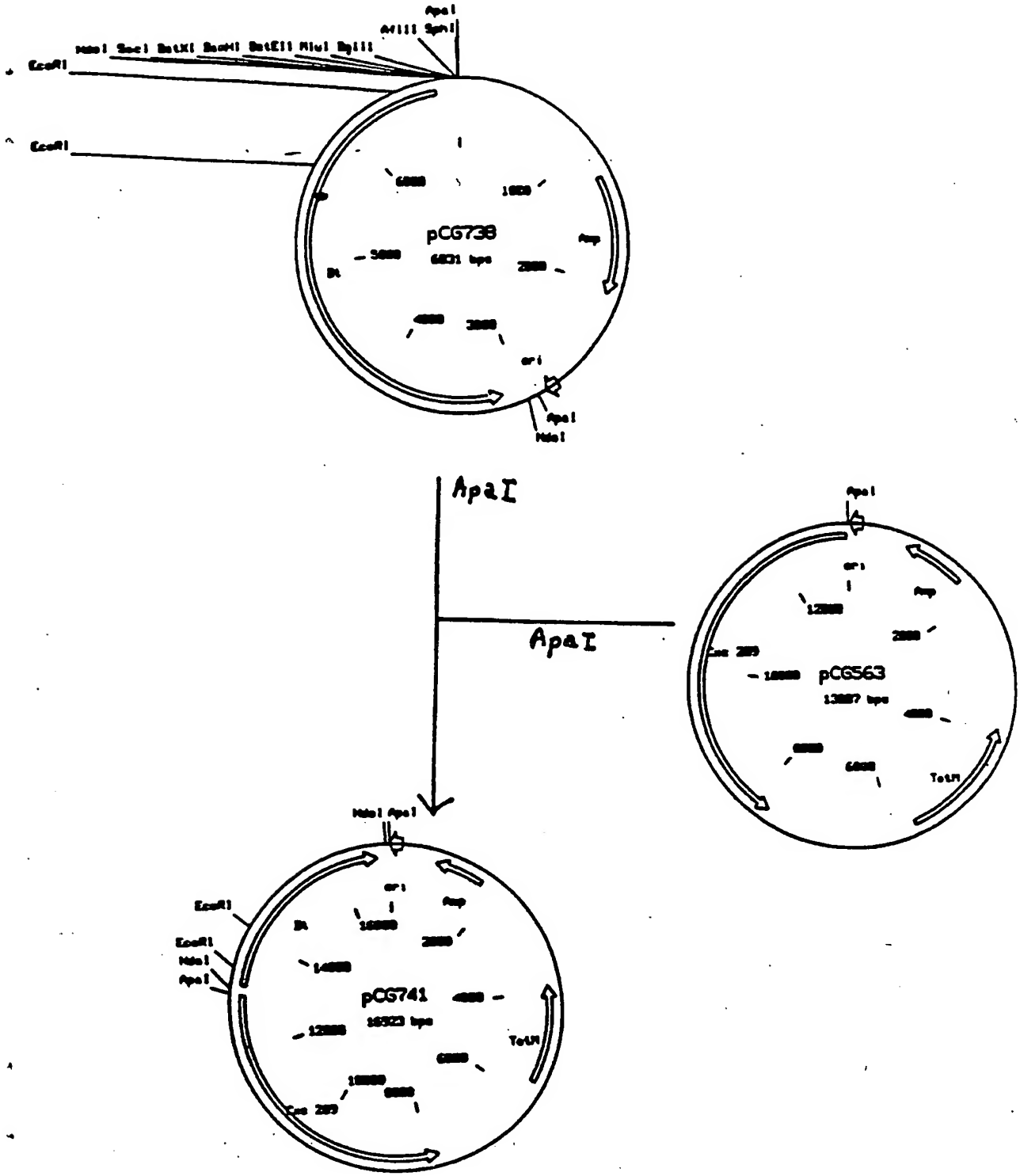


Figure 13

So. . of Probes.

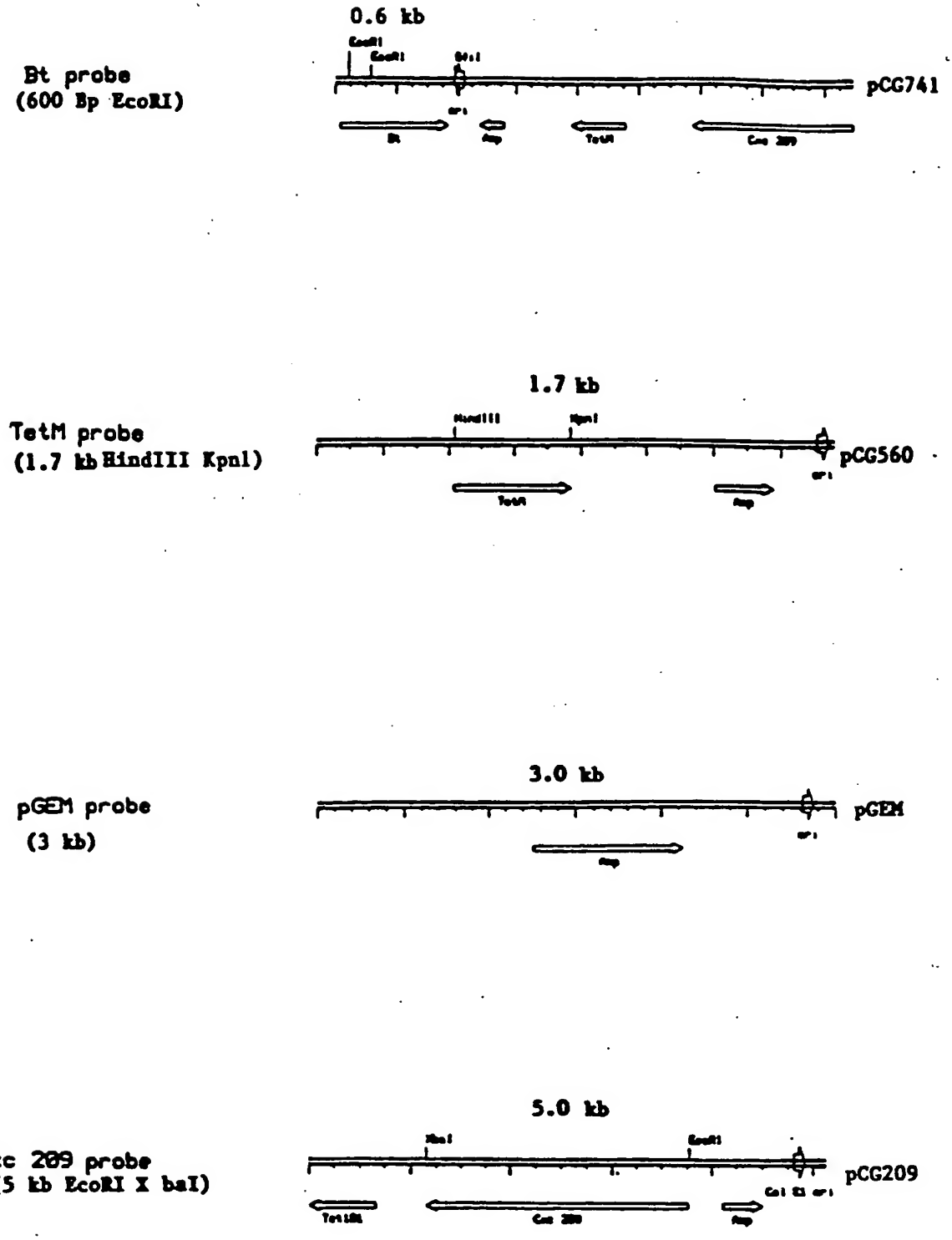


Figure 14

Restriction enzyme sites used for southern analysis of pCG741 in MDR1.586.

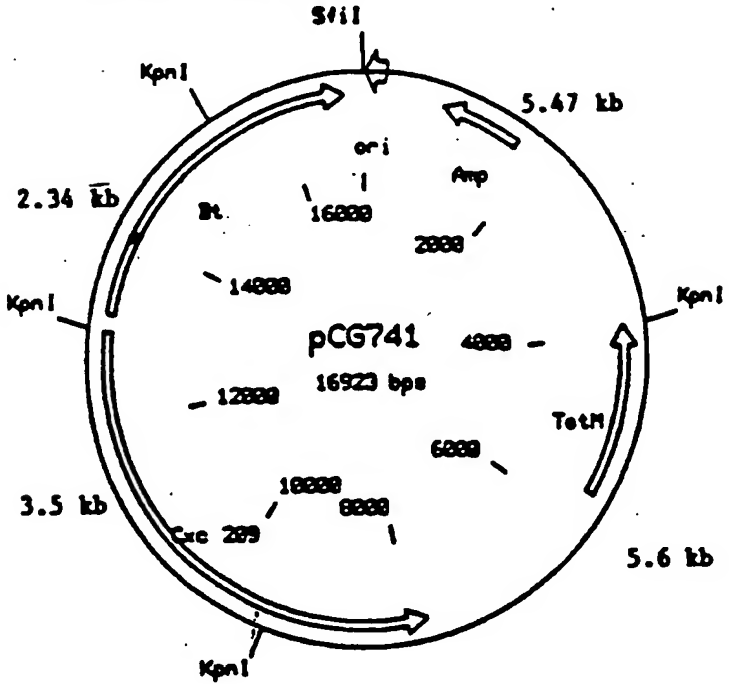
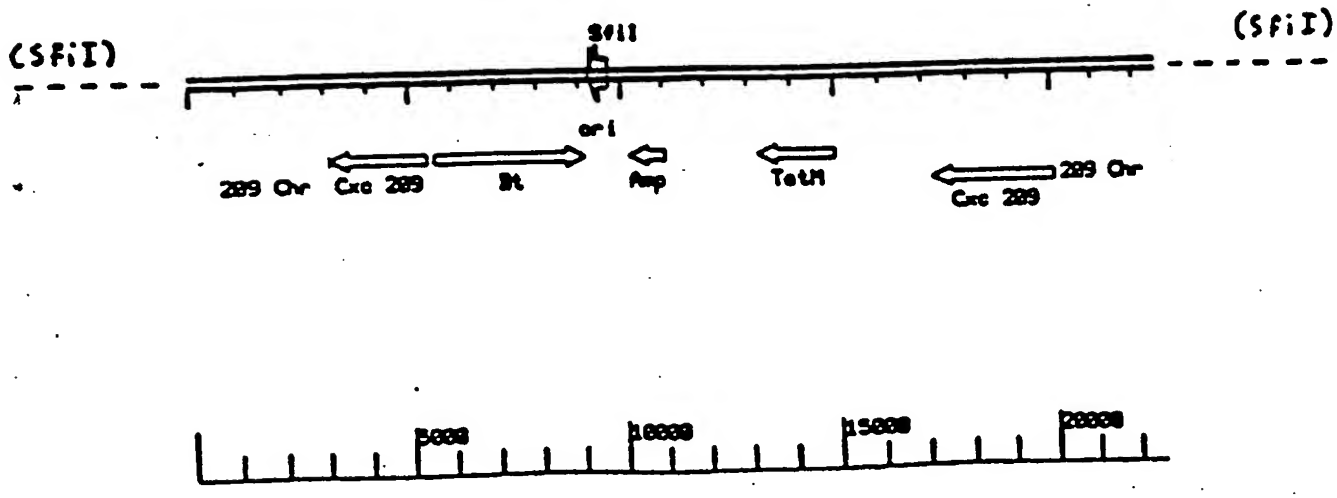
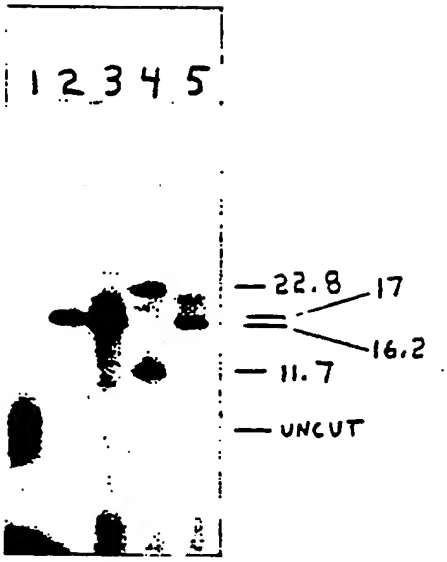


Figure 15

Chromosomal Integrate Structure



SfiI Digest of DNA Samples: Southern Analysis



Lane 1 is 5 ng uncut pCG741 pure plasmid; Lane 2 is 5 ng pCG741 cut with SfiI; lane 3 is a mix of 5 ng pCG741 and 2 µg MDE1 chromosomal DNA cut together with SfiI; lane 4 contains 2 µg MDR1.586 chromosomal DNA cut with SfiI; lane 5 contains 2 µg MDE1 chromosomal DNA cut with SfiI. The probe is Cxc 209 DNA (Fig. 9). The resolving gel is 0.4% Agarose/TAE. Molecular weights of fragments are shown on right of figure, computed by reference to ethidium bromide stained markers.

Figure 16

KpnI Digests of DNA Samples: Southern Analysis.

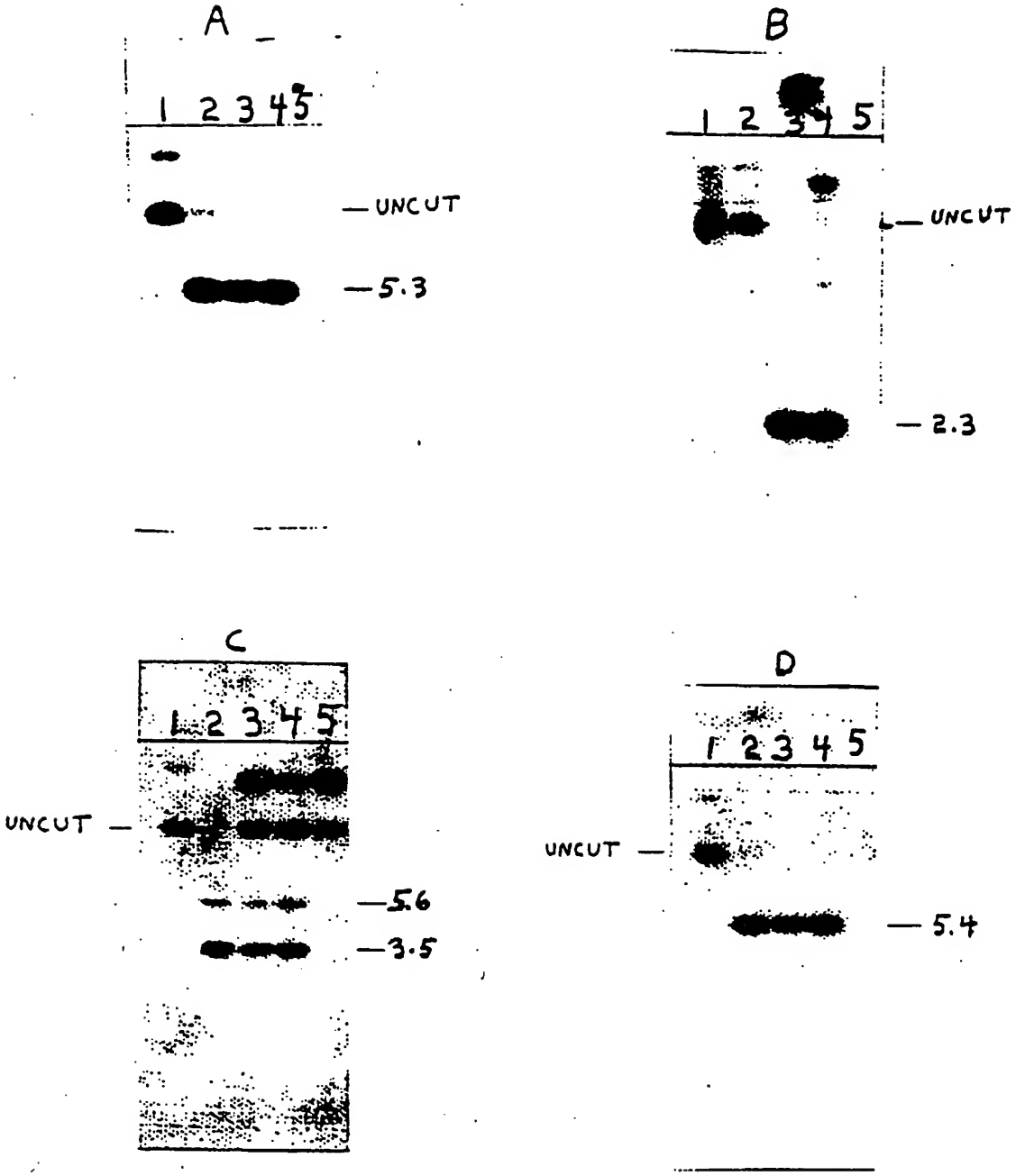


Figure 17

Lane 1 is 5 ng uncut pCG741; lane 2 is 5 ng pCG741 cut with KpnI; lane 3 is a mixture of 5 ng pCG741 and 2 µg MDE1 DNA cut with KpnI; lane 4 is 2 µg MDR1.586 cut with KpnI; lane 5 is 2 µg MDE1 cut with KpnI.

Probes (Fig. 9) are: (A) = pGEM; (B) = Bt; (C) = Cxc209; (D) = tetM. Resolving gel is 1% agarose/TAE. Molecular weights of fragments are given in kb on right of figure. Lane 2 (b) did not receive enough KpnI to cut.

Population dynamics of new construction MDR1.586 and control strains in the basal shoot (2 weeks) and the basal internode (6 weeks and 10 weeks) of greenhouse grown corn.

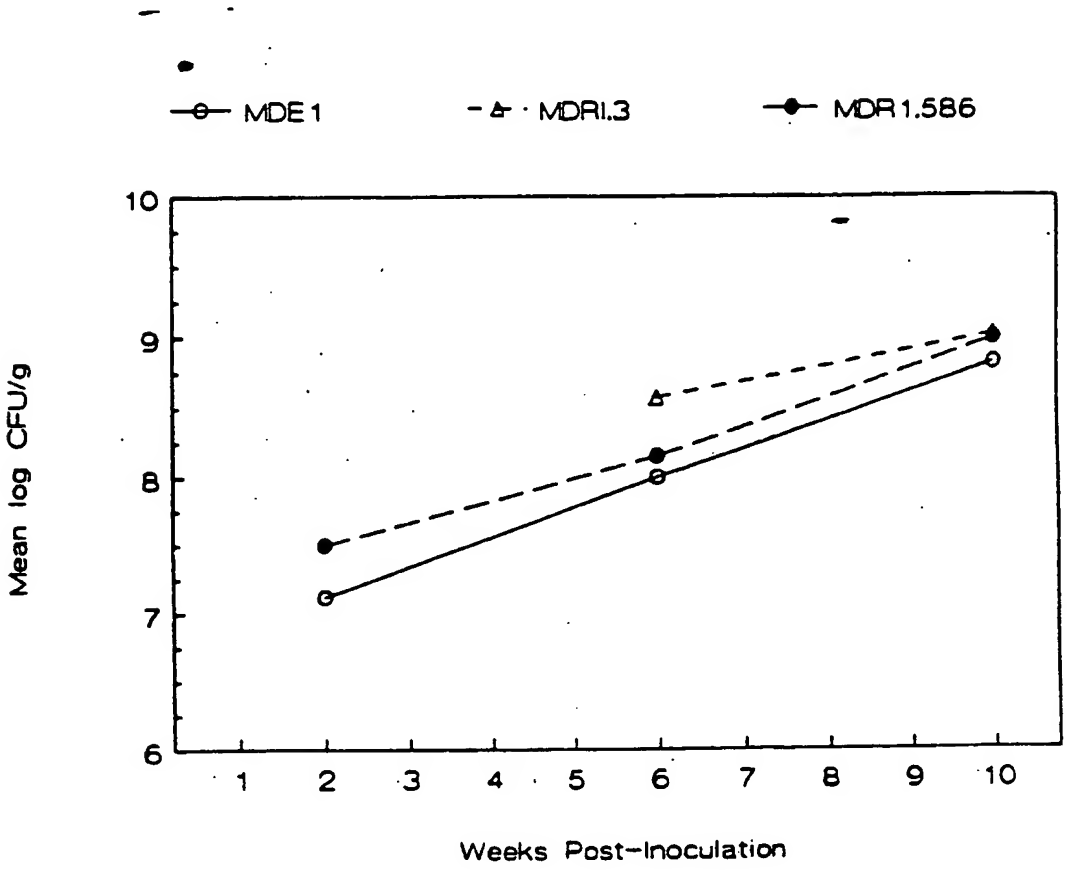
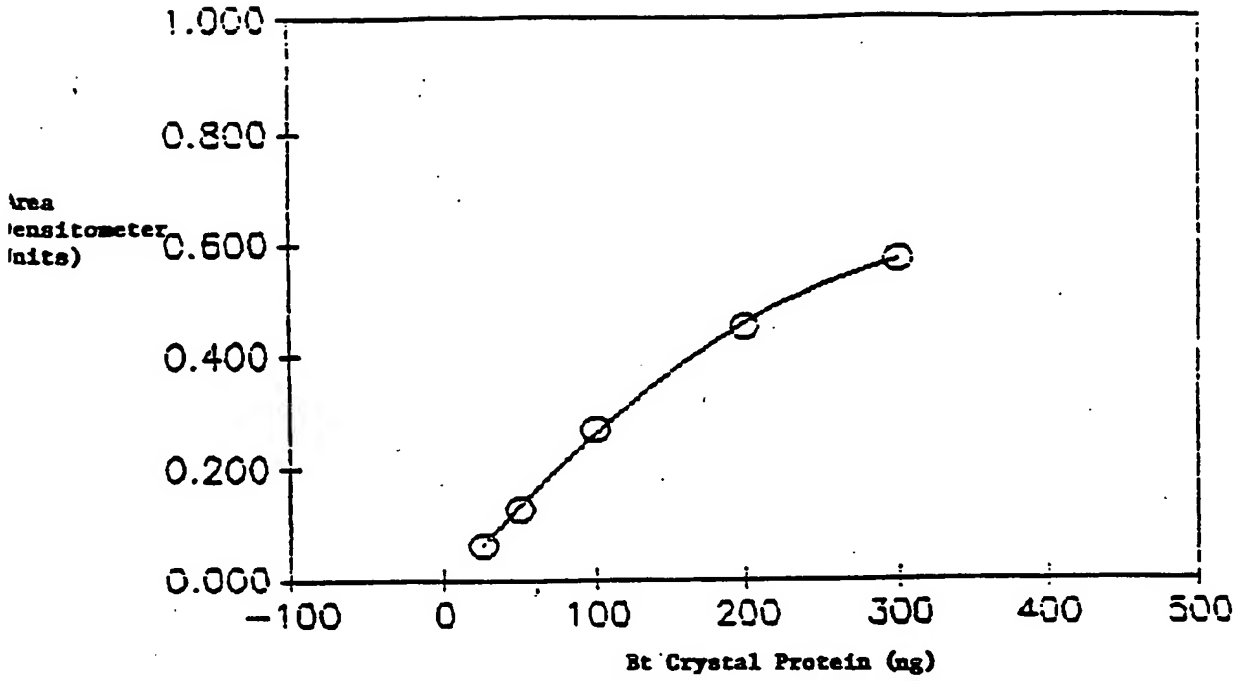
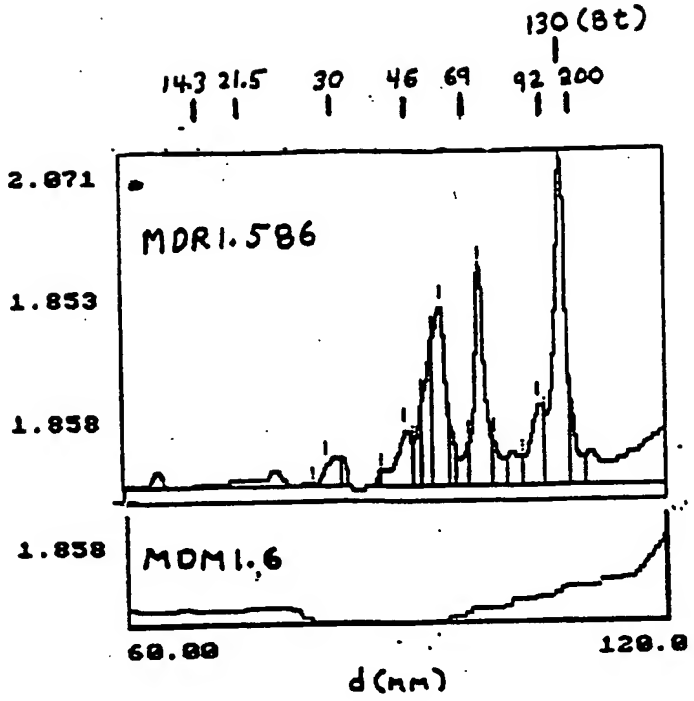


Figure 18



Standard curve generated by scanning densitometry of immunovisualized Western blot. The standards are the indicated quantities of Bt crystal protein solubilized in SDS sample buffer and resolved in a 4-20% SDS polyacrylamide gel. The protein was electrophoretically blotted onto Immobilon paper and the 130 kd protoxin visualized by an anti-Bt (goat):anti-goat (rabbit)(alkaline phosphatase conjugant) sandwich.

Figure 19



Densitometry of Western Blots.

Densitometric trace of immunovisualized Bt antigen of strain MDR1.586 (top) and control extract MDM1.6 (bottom). Molecular weight marker positions are listed by their Kdalton values (top), and the position of the 130 Kdalton Bt crystal protein is also shown.

Figure 20